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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/54, 15/82, A01H 5/00 — A1

(11) International Publication Number: **WO 00/18930**

(43) International Publication Date: 6 April 2000 (06.04.00)

(21) International Application Number: PCT/GB99/03021

(22) International Filing Date: 13 September 1999 (13.09.99)

(30) Priority Data:
9820970.3 25 September 1998 (25.09.98) GB

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PLANT PROMOTER

(57) Abstract

The invention relates to the gene promoter region for the 27 kD subunit of the glutathione–S–transferase, isoform II, or a deleted fragment thereof which retains the activity of the gene promoter region. Such promoters are constitutive in tubers and can therefore be used to control the expression of a gene to prevent or inhibit sprouting of tubers.

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PLANT PROMOTER

The present invention relates to a promoter. In particular, the present invention relates to a promoter which is constitutive in tubers and in the stems of plants.

More particularly, the present invention relates to a method of constitutively expressing a target gene in a storage organ or stem of a plant.

Gene expression is controlled by various regulatory components, including nucleic acid and protein elements. In particular, gene expression is controlled by a region commonly referred to as the "promoter" which lies upstream (5') of the protein encoding region. A promoter may be constitutive or tissue-specific, developmentally-regulated and/or inducible.

Within the promoter region there are several domains which are necessary for full function of the promoter. The first of these domains lies immediately upstream of the structural gene and forms the "core promoter region" containing consensus sequences, normally 70 base pairs immediately upstream of the gene. The core promoter region contains the characteristic CAAT and TATA boxes plus surrounding sequences, and represents a transcription initiation sequence which defines the transcription start point for the structural gene. The precise length of the core promoter region is indefinite but it is usually well-recognisable. Such a region is normally present, with some variation, in all promoters. The base sequences lying between the various well-characterised "boxes" appear to be of lesser importance.

The presence of the core promoter region defines a sequence as being a promoter: if the region is absent, the promoter is non-functional. Furthermore, the core promoter region is insufficient to provide full promoter activity. A series of regulatory sequences upstream of the core constitute the remainder of the promoter. The regulatory sequences determine expression level, the spatial and temporal pattern of expression and, for an important subset of promoters, expression under inductive conditions (regulation by external factors such as light, temperature, chemicals, hormones).

Manipulation of crop plants to alter and/or improve phenotypic characteristics (such as productivity or quality) requires the expression of heterologous genes in plant tissues. Such genetic manipulation therefore relies on the availability of means to drive and to control gene expression as required; for example, on the availability and use of suitable promoters

which are effective in plants and which regulate gene expression so as to give the desired effect(s) in the transgenic plant. It is advantageous to have the choice of a variety of different promoters so that the most suitable promoter may be selected for a particular gene, construct, cell, tissue, plant or environment.

Promoters (and other regulatory components) from bacteria, viruses, fungi and plants have been used to control gene expression in plant cells. Numerous plant transformation experiments using DNA constructs comprising various promoter sequences fused to various foreign genes (for example, bacterial marker genes) have led to the identification of useful promoter sequences. It has been demonstrated that sequences up to 500-1000 bases in most instances are sufficient to allow for the regulated expression of foreign genes. However, it has also been shown that sequences much longer than 1 kb may have useful features which permit high levels of gene expression in transgenic plants. A range of naturally-occurring promoters are known to be operative in plants and have been used to drive the expression of heterologous (both foreign and endogenous) genes in plants: for example, the constitutive 35S cauliflower mosaic virus promoter, the ripening-enhanced tomato polygalacturonase promoter (Bird et al, 1988, Plant Molecular Biology, 11:651-662), the E8 promoter (Diekman & Fischer, 1988, EMBO, 7:3315-3320) and the fruit specific 2A11 promoter (Pear et al, 1989, Plant Molecular Biology, 13:639-651) and many others.

Two principal methods for the control of expression are known, viz.: overexpression and underexpression. Overexpression is achieved by insertion of one or more than one extra copies of the selected gene. It is, however, not unknown for plants or their progeny, originally transformed with one or more than one extra copy of a nucleotide sequence, to exhibit the effects of underexpression as well as overexpression.

For underexpression there are two principle methods which are commonly referred to in the art as "antisense downregulation" and "sense downregulation" (sense downregulation is also referred to as "cosuppression"). Generically these processes are referred to as "gene silencing". Both of these methods lead to an inhibition of expression of the target gene. Other lesser used methods involve modification of the genetic control elements, the promoter and control sequences, to achieve greater or lesser expression of an inserted gene.

There is no reason to doubt the operability of sense/cosuppression technology. It is well established, used routinely in laboratories around the world and products in which it is used are on the market.

Gene control by any of these methods requires the insertion of a selected gene or genes into plant material which can be regenerated into plants. This transformation process can be performed via a number of methods, for example: the *Agrobacterium*-mediated transformation method.

In summary, the requirements for both sense and antisense technology are known and the methods by which the required sequences may be introduced are known. What remains then is to identify genes whose regulation will be expected to have a desired effect, isolate them or isolate a fragment of sufficiently effective length, construct a chimeric gene in which the effective fragment is inserted between promoter and termination signals, and insert the construct into cells of the target plant species by transformation. Whole plants may then be regenerated from the transformed cells.

A skilled person may use a suitable expression cassette (incorporating one or more promoters and other components) to regulate gene expression in the desired manner (for example, by enhancing or reducing expression in certain tissues or at certain developmental stages). The ability to choose a suitable promoter from a range of promoters having differing activity profiles is thus important.

Potato tubers are of major economic importance. They represent a carbohydrate resource for many diets and are used as a basis for a variety of processed products. Besides starch, tubers contain high-quality proteins, substantial amounts of vitamins, minerals and trace elements.

Potatoes cannot be grown throughout the year in most growing regions and it is therefore desirable to store them when they are not in the growing season. One of the potentially most damaging phenomena during storage is premature sprouting. Current methods of sprouting suppression include cooling and chemical sprout suppressants.

Cooling, usually done in Northern Europe by ventilation with air at ambient temperature is one of the methods to inhibit sprouting. Apart from the associated costs, longer term cooling at 4 °C gives rise to the problems of cold sweetening and melanisation (darkening).

The use of chemical sprouting suppressants is currently the only possibility for inhibiting sprouting in potatoes destined for processing and fresh consumption, since low temperature storage leads to unacceptable accumulation of reducing sugars. It would also be desirable to obviate the use of chemicals and their associated costs and inconvenience. There is therefore a real need for an alternative method of controlling sprouting in vegetative storage organs such as tubers. An alternative approach to delay sprouting would be the use of transgenic plants with a prolonged quiescence period.

Sprouting of potato tubers involves several independent steps which might be targets for genetic engineering. The first step is the mobilisation of reserves, mainly starch. Starch breakdown occurs in amyloplasts. After transfer into the cytosol the produced hexoses and hexose-phosphates are distributed between glycolysis and sucrose synthesis. The third step is the formation of sucrose in the cytosol. Sucrose synthesis is energy dependent thus glycolysis and respiration are required. The fourth step is the transport of sucrose to the developing sprout. Finally, the imported sucrose is utilised in the sprout to support growth and development.

We have now developed an approach to repress sprouting in tubers where a sprout suppressant gene is driven throughout all stages of tuber dormancy by a gene promoter which is constitutive in vegetative storage organs and stems of plants such as potatoes.

The present invention therefore seeks to provide a promoter which is expressed constitutively throughout development, storage and sprouting of a vegetative storage organ and in the stem of a plant. Such a promoter could be used in the suppression of sprouting of tubers and more particularly in potato tubers.

According to the present invention, there is provided a method of constitutively expressing a target gene in a vegetative storage organ or stem of a plant comprising incorporating, preferably stably incorporating, into the genome of the plant a DNA construct comprising a first polynucleotide sequence comprising the gene promoter region for the 27 kD subunit of the glutathione-S-transferase, isoform II, or a deleted fragment thereof which retains the activity of the gene promoter region, operably linked to and controlling a target gene sequence.

Preferably, the DNA construct further comprises a second polynucleotide sequence

comprising at least one DNA sequence operably linked to and under the control of an inducible promoter region.

Preferably, expression of the target gene results in the suppression of sprouting of the storage organ.

Preferably, the suppression of sprouting is neutralised by inducing expression of the second polynucleotide sequence.

Preferably, the second polynucleotide sequence is a sense, antisense or partial sense sequence corresponding to said first polynucleotide sequence or a DNA sequence capable of causing suppression of the protein encoded by the target gene.

Preferably, the first and second polynucleotide sequences further comprise a transcription terminator region.

Preferably, the gene promoter is the maize 27 kD subunit of the glutathione-S-transferase, isoform II gene promoter (GST promoter, Figure 8 and SEQ ID NO:1), although similar promoters from other organisms may also be used. The GST promoter is described in our earlier patent applications publication Nos. WO 93/01294 and WO 97/11189. In addition, it is possible to use variants of the GST promoter of SEQ ID NO: 1 which are substantially homologous thereto.

When comparing nucleic acid sequences for the purposes of determining the degree of homology or identity one can use programs such as BESTFIT and GAP (both from the Wisconsin Genetics Computer Group (GCG) software package) BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables sequences to be aligned along their whole length and finds the optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention compare when discussing homology of nucleic acid sequences, the comparison is made by alignment of the sequences along their whole length.

Preferably, sequences which have substantial homology have at least 50% sequence homology, desirably at least 70% sequence homology and more desirably at least 80%, 90% or at least 95% sequence homology, in increasing order of preference, with said sequences. In some cases the sequence homology may be 99% or above.

Desirably, the term "substantial identity" indicates that said sequence has a greater degree of identity with any of the sequences described herein than with prior art nucleic acid sequences.

Although, as mentioned above, variants of SEQ ID NO: 1 can be used as the promoter sequence, it is greatly preferred that the sequence from about base 3590 to 3822 is substantially conserved, or at least not less than 80% homologous with the SEQ ID NO: 1.

It is also possible to use a deleted fragment of the promoter, provided that fragment retains the activity of the gene promoter region. Examples of such fragments are described in our earlier patent application published as WO 97/11189 and include the following regions of the 27 kD subunit of glutathione-S-transferase:

a sequence comprising 897 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase;

a sequence comprising 570 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase; or

a sequence comprising 378 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

The transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase is defined in WO 93/01294 and WO 97/11189 as being at position 3701 of SEQ ID NO: 1.

One useful fragment comprising 570 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase is the 693 base fragment shown in Figure 9 (SEQ ID NO: 2).

Preferably, the storage organ is a tuber.

Preferably, the plant is a potato plant.

According to a preferred embodiment of the present invention is there is provided a method of constitutively expressing a target gene in a storage organ or stem of a plant comprising incorporating, preferably stably incorporating, into the genome of the plant a DNA construct comprising a first polynucleotide sequence comprising the gene promoter region for the 27 kD subunit of the glutathione-S-transferase, isoform II, or a deleted fragment thereof which retains the activity of the gene promoter region, operably linked to

and controlling a target gene sequence, the DNA construct further comprising a second polynucleotide sequence comprising at least one DNA sequence operably linked to and under the control of an inducible promoter region.

The term "transgenic" in relation to the present invention does not include a wild type regulator promoter in its natural environment in combination with its associated functional gene in its natural environment.

The term "target gene" with reference to the present invention means any gene of interest. A target gene can be any gene that is either foreign or natural to the plant in question.

The term "construct" - which is synonymous with terms such as "cassette", "hybrid" and "conjugate" - includes a target gene directly or indirectly attached to the regulator promoter, such as to form a cassette. An example of indirect attachment is the provision of a suitable spacer group such as an intron sequence intermediate the promoter and the target gene. The same is true for "fused" in relation to the present invention which includes direct or indirect attachment. Such constructs also include plasmids and phage which are suitable for transforming a cell of interest.

The term "inducible promoter" includes promoters which may be induced chemically. The use of a promoter sequence which is controlled by the application of an external chemical stimulus is most especially preferred. The external chemical stimulus is preferably an agriculturally acceptable chemical, the use of which is compatible with agricultural practice and is not detrimental to plants or mammals.

References to "potatoes" also include other plants of the potato family, such as sweet potatoes and similar plants.

The inducible promoter region most preferably comprises an inducible switch promoter system such as, for example, a two component system such as the *alcA/alcR* gene switch promoter system described in our published International Publication No. WO 93/21334 and the ecdysone switch system as described in our International Publication No. WO 96/37609, the teachings of which are incorporated herein by reference. Such promoter systems are herein referred to as "switch promoters". The switch chemicals used in conjunction with the switch promoters are agriculturally acceptable chemicals making this system particularly useful in the method of the present invention. In the case of the

alcA/alcR promoter switch system, the preferred chemical inducer is ethanol in either liquid or vapour form.

Suitable transcription terminators which may be used are also well known in the art and include, for example, the nopaline synthase terminator and octopine synthase terminators.

The DNA sequences in the DNA construct may be endogenous or heterologous with respect to the transformed host.

Examples of target DNA sequences which may be used in the present invention to control sprouting include those DNA sequences coding for proteins involved in the mobilisation of reserves during dormancy e.g.starch breakdown, i.e. starch phosphorylase, amylase (e.g. α or β amylase) and maltase; e.g. in glycolysis and subsequent metabolism e.g. phosphofructokinase, hexokinase; in sucrose biosynthesis e.g. sucrose synthase; in the transport of reserves during dormancy such as in phloem loading e.g. ATPase; in long distance phloem transport and in phloem unloading e.g.inorganic pyrophosphorylase (iPPase); and in the utilisation of reserves during dormancy such as in assimilate breakdown e.g. the breakdown of sucrose in the growing sprout, i.e. invertase; and in the utilisation of assimilates e.g. utilisation of sucrose-derived metabolites, in the provision of energy required for sprout formation e.g.adenine nucleotide translocator (ANT) and malate oxoglutarate translocator (MOT) and inhibitors thereof such as uncoupling proteins. Examples of useful DNA sequences also include any other sequences which are involved in potato sprouting.

Examples of preferred target DNA sequences which may be used in the method of the present invention to control sprouting include those resulting in the production of sense, anti-sense or partial sense sequence(s) to, and/or coding for, proteins involved in the mobilisation and/or utilisation of sucrose, in potato sprouting and in mitochondrial function, such as in respiration.

Examples of particularly preferred target DNA sequences include those coding for an invertase derived from plant, bacterial or fungal sources e.g. from yeast, a pyrophosphatase derived from plant, bacterial or fungal sources and proteins involved in mitochondrial function such as adenosine nucleotide translocator protein (ANT) or mitochondrial oxoglutarate translocator (MOT) derived from plant, bacterial or fungal sources.

Suppression of sprouting may be achieved in a variety of ways. The first DNA sequence(s) may be expressed during dormancy of the vegetative storage organ and then down-regulated when sprouting is desired. When sprouting is desired, expression of the second DNA sequence(s) is turned on leading to down-regulation of the first DNA sequence and consequently to restoration of sprouting.

Down-regulation of a desired target DNA sequence(s) may be achieved using methods well known in the art such as, for example, by use of repressor proteins, sense, anti-sense, partial-sense, and expression of a complementary protein. Examples of suitable operator/repressor systems include for example the lac, tet or lambda 434 systems and mutants thereof such as the Lac IΔ His mutant (Lehming, N., Sartoris, J., Niemoeller, M., Genenger, G., v. Wilcken-Bergman, B. and Muller-Hill, Benno (1987), EMBO J. 6(10) 3145-3153 - where the mutant has a change in amino acid 17 of Lac I altering tyrosine for histidine). Alternatively, an Amplicon™ may be used to down-regulate genes (Angell, S.M., Baulcombe, D.C., (1997) 16, 3675-3684). In this regard, the cDNA of replicating potato virus (PVX) RNA which has a transgene inserted therein is used whereby transiently expressed RNA sharing homology with the transgene is suppressed.

Alternatively, expression of the DNA sequence(s) in the first polynucleotide sequence may result in the production of a sense, anti-sense or partial-sense sequence(s) which acts to suppress a gene involved in sprouting or in the expression of an Amplicon™. In this case sprouting is restored by switching on expression of the DNA sequence(s) in the second polynucleotide sequence which results in production of the protein or a corresponding protein to that, the production of which was suppressed by the sense, anti-sense or partial-sense sequence(s) in the first DNA sequence. Sprouting may also be restored by means of a suitable operator/repressor system.

Where either or both of the polynucleotide sequences in the construct comprise more than one DNA sequence it is preferable that they are not identical to avoid any co-suppression effects.

The DNA sequence(s) in the second polynucleotide sequence of the construct is under the control of an inducible promoter region.

Suitable transcription terminators which may be used are also well known in the art and include for example the nopaline synthase terminator and octopine synthase terminators.

The inducible promoter region for use in the method of the present invention is preferably the *alcA/ alcR* promoter switch system. Restoration of sprouting is preferably achieved using switchable antisense or switchable sense or partial sense methods as is described more fully herein or alternatively by use of an Amplicon™ or by means of a suitable operator/repressor system. Down-regulation of gene activity due to partial sense co-suppression is described in our International Patent Application No. WO 91/08299 the teachings of which are incorporated herein and this may be avoided if necessary by using gene sequences derived from different organisms.

An advantage of the present invention is that expression is maintained throughout tuber development to tuber sprouting.

The GST-II-27 promoter was originally isolated in maize and it was shown to be inducible in leaf tissue as described in our International publication Nos. WO 90/08826 and WO 93/01294. The genomic DNA sequence encoding the gene promoter for the 27kD subunit of the glutathione-S-transferase, isoform II, enzyme (GST-II-27), containing the nucleotide sequence shown in Figures 8 and 9 herewith was deposited as plasmid pGIE7 within *E.coli* strain XLI-Blue in the National Collections of Industrial and Marine Bacteria (NCIMB) under accession number NCIMB 40426. Furthermore, although expression of the GST-II-27 promoter in root tissue is known to be constitutive (see Reference: Holt, D. C., Lay, V. J., Clarke, E. D., Dinsmore, A., Jepson, I., Bright, S. W. J., and Greenland, A. J. (1995) Characterisation of the safener-induced glutathione S-transferase isoform II from maize. Planta **196**, 295-302), constitutive expression of the GST-II-27 promoter in the stem and tuber organs was an unexpected finding.

Tubers are not root tissue, but are initiated on underground stems called stolons. Tuberisation starts in the youngest elongating internode, just below the stolon apex. The stolon elongation stops and radial growth begins by cell expansion and then division. Starch accumulates in the developing tuber and storage glycoproteins are formed. A tuber may be described as a modified stem, as it has nodes, internodes and axillary buds.

In summary, the GST-II-27 promoter can be used as a constitutive promoter in stems and tubers as it is expressed in the absence of any inducer being applied to the plant. It will not be expressed in leaf tissue unless a GST-II-27 inducer is applied. Applications of such a constitutive promoter include expression of a tuber sprout suppressant gene from the GST-II-

27 promoter, which could be reversed, for example, by antisense or co-suppression from an inducible promoter.

The DNA construct comprising the gene promoter region for the 27 kD subunit of the glutathione-S-transferase, isoform II, or a deleted fragment thereof which retains the activity of the gene promoter region, operably linked to and controlling a target gene sequence comprises a further aspect of the invention.

In the DNA construct of the invention, the target gene will generally be a gene whose expression results in the suppression of sprouting in the vegetative storage organ of a plant such as a potato. Preferred types of sprouting suppression genes are as discussed above in relation to the first aspect of the invention.

A particularly suitable gene promoter for use in the DNA construct of the invention is the maize 27 kD subunit of the glutathione-S-transferase, isoform II gene promoter (SEQ ID NO: 1) or a sequence substantially homologous thereto as defined above. Fragments of the 27 kD subunit of the glutathione-S-transferase, isoform II gene promoter which retain the activity of the gene promoter region may also be used. As already mentioned, such fragments include:

a sequence comprising 897 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase;

a sequence comprising 570 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase; or

a sequence comprising 378 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase.

The transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase is defined in WO 93/01294 and WO 97/11189 as being at position 3701 of SEQ ID NO: 1.

One useful fragment comprising 570 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase is the 693 base fragment shown in Figure 9 (SEQ ID NO: 2).

It is preferred that the DNA construct of the invention also comprises a second polynucleotide sequence comprising at least one DNA sequence operably linked to and under the control of an inducible promoter region. As already mentioned in relation to the method

of the first aspect of the invention, the second polynucleotide sequence may be a sense, antisense or partial sense sequence corresponding to said first polynucleotide sequence or a DNA sequence capable of causing suppression of the protein encoded by the target gene.

The construct may also comprise a transcription terminator region.

The DNA construct of the invention may be used to transform a plant of the potato family in order to suppress sprouting of the tubers. Therefore, in a further aspect of the invention, there is provided potato plant germ plasm comprising a DNA construct as defined above.

The invention also provides a potato plant, potato seed or potato plant cell comprising a DNA construct as defined above.

Furthermore, in yet a further aspect of the present invention, there is provided a method for preventing or inhibiting sprouting in a potato tuber, the method comprising causing the tuber to express a target sequence as defined above under the control of the gene promoter region for the 27 kD subunit of the glutathione-S-transferase, isoform II, or a deleted fragment thereof which retains the activity of the gene promoter region.

Various preferred features and embodiments of the present invention will now be described by way of non-limiting examples with reference to the accompanying Figures:-

Figure 1A is a graph showing induction of the GST promoter in *Solanum tuberosum* variety *solara* leaf tissue.

Figure 1B is a graph showing a further root drench induction of the GST promoter.

Figure 2 is a graph showing a time course experiment using GST:GUS line 6 plants.

Figure 3A is a graph showing a fluorometric GUS assay of tissues of 6 week old plants root drenched with safener, 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone.

Figure 3B is a photograph showing histochemical staining with X-gluc (5-Bromo-4-Chloro-3-Indolyl-β-D-Glucuronide with tissue pieces from plants used in the fluorometric assay.

Figure 3C is a photograph showing histochemical staining on uninduced and induced stained stem sections.

Figure 3D is a photograph showing a longitudinal section through the stem of safener a induced plant stained with X-gluc.

Figure 4 is a photograph showing a transverse section through uninduced 6 week old potato stem stained with X-gluc.

Figure 5A is a photograph showing histochemical staining of tubers from 4 month old plants harvested before and after root drenching with 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone safener and stained with X-gluc. Tubers shown were obtained from wild type, 35SGUS and from transgenic plants which contained a 3822 base pair GST-27 promoter fused to a GUS reporter gene. Tubers from two of the transgenic lines are shown here. The lines are known as G6 and G44. These were identified as high expressors of GST-27:GUS in the primary transformants. This was achieved by treating the primary transformants with safener in a root drench and assaying leaf tissue by fluorometric means for GUS protein (see Figure 1B).

Figure 5B is a graph showing a fluorometric assay of tubers from uninduced and induced plants.

Figure 6A is a photograph showing the histochemical staining of 6 month old tubers from storage from lines G6 and G44 and a wild type negative control.

Figure 6B is a photograph showing histochemically stained 8 month old tubers at the sprouting stage.

Figures 7A and 7B are photographs showing stained tubers throughout the potato lifr-cycle - 2 months, 4 months, 6 months and 8 months compared with wild type and CaMV 35S controls.

Figure 8 shows the full GST promoter sequence comprising 3822 base pairs. The part which comprises the deleted GST promoter sequence is highlighted.

Figure 9 shows a deleted GST promoter sequence comprising 693 bases.

Figure 10 shows a construct comprising the full GST promoter sequence.

Figure 11 shows a construct comprising a deleted GST promoter sequence.

Figure 12 is a set of photographs showing slices from tubers from transgenic plants which contained the fragment of SEQ ID NO: 2 of the GST-27 promoter fused to a GUS reporter gene compared with a slice from a tuber from line G6. The slices are histochemically stained with X-gluc to test for GUS expression.

Figure 13 is is a set of photographs showing two sets of slices from tubers from transgenic plants which containing the full length 3.8 kb GST promoter fused to a GUS

reporter gene compared with a slice from a wild type tuber. The first set of slices is uninduced and the second set has been treated with the chemical safener 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone applied at the rate of 20mg/40ml (0.5 g/l). The slices are histochemically stained with X-gluc to test for GUS expression.

Figure 14 is a plot showing GST and patatin controlled expression of GUS throughout the life cycle of various plants.

Figure 15 is a plot showing a comparison of GUS activity in tuber tissue over time for a variety of plants, including wild type, CaMV 35S, patatin:GUS and GST:GUS plants.

Figures 16a to 16e is a series of plots showing expression of GUS throughout the tuber life cycle for a selection of plants as follows: wild type (Figure 16a), G6 (Figure 16b) 842-06 (Figure 16c), 842-07 (Figure 16d) and 842-03 (Figure 16e). The plants designated 842 contain the patatin promoter.

Figure 17 is a series of photographs 12 showing slices from tubers from wild type and transgenic plants containing a promoter fused to a GUS reporter gene. The slices were taken at various times during the growth and storage of the plants and are histochemically stained with X-gluc to test for GUS expression.

Figure 18 is a photograph showing the size of the sprouts on the tubers of lines GST-II-27:GUS 6, GST-II-27:GUS 44, 35S:GUS (410), Patatin:GUS (842) and wild type. The tubers were removed from storage at 47 weeks and contained sprouts of between 0.5 and 3cm in length. The tubers were sampled for histochemical and fluorometric GUS analysis.

Figure 19 is a photograph showing a slice of a GST-II-27:GUS line 6 tuber sampled at 47 weeks. The tuber was sprouting. The slice was histochemically stained using X-gluc to show expression of GUS activity at sprouting.

Example 1

A construct comprising the 3822 base pair 5' region of the GST-II-27 promoter fused to the GUS reporter gene and the Nos 3' termination region was transformed into potato. Figures 10 and 11 show constructs comprising both the full and deleted GST promoters.

Standard recombinant DNA methods were adopted in the construction of plasmid vectors. A reporter gene construct containing a *GST-27* 3.8 kb *EcoRI-Nde* I 5' flanking region from pG1E7 was blunted ended and ligated into the *Sma* I site of the *Agrobacterium* Ti vector

pBI101. The *Nde* I site, which lies at the predicted translation start codon of *GST-27* was destroyed after blunting. This formed a convenient point for fusion with the *E.coli UidA* gene, encoding b-glucuronidase (GUS) in pBI101. The structure of the resultant chimeric reporter gene construct pGSTTAK was verified by restriction and sequence analysis.

To generate the deleted promoter of SEQ ID NO: 2, a PCR product was synthesised using a pAI5 oligo at the 5' end and a pAI2 oligo at the 3' end of the GST-27 promoter fragment. The 5' pAI5 oligo had the sequence GCGGCAAGCTTAATATGTGATGATA and contained a HindIII site. The pAI2 oligo had the sequence TGCCTGCTGCAGCTGCTACTTAT, and hybridised to a promoter sequence which contained a Pst1 site. The purified PCR fragment was digested with HindIII and Pst1 and ligated into a HindIII-Pst1 vector fragment of the pGSTTAK clone

Other constructs were also constructed using deleted fragments of the GST-II-27 promoter. Such deleted fragments are described in our International Publication No. WO 97/11189.

Using plasmid pGST::GUS direct transformation of *Agrobacterium tumefaciens* strain C58C1:pGV2260 was carried out as described by Höfgen and Willmitzer (1988) (J. loc cit). Potato transformation (*solara*) using *Agrobacterium*-mediated gene transfer was performed as described by Rocha-Sosa et al. (1989) (J. loc cit).

Example 2

Analysis of the GST -27 expression patterns in these potato plants was carried out. Tissue from various plant parts were taken from untreated plants and plants treated with the herbicide safener, *N,N*-diallyl 1-2,2-dichoroacetamide. In particular, the GST:GUS construct was induced in *Solanum tuberosum* variety *solara* via root drench with chemical safener, *N,N*-diallyl 1-2,2-dichoroacetamide.

Results from induction in leaf tissue are shown in Figure 1A. Primary analysis shows a clear induction of GST:GUS after application of *N,N*-diallyl 1-2,2-dichoroacetamide safener by addition to the growth media. It was found that a concentration of 10 % safener produced the greatest level of safener induction.

A more thorough repetition of root drench induction with a different safener, 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone was then carried out. Leaf tissue results were compared with wild type and CaMV 35S plant leaf tissue. Fold inductions are shown in

Figure 1B. Two plant lines show high fold inductions after safener application and high levels of GST:GUS expression.

Example 3

A time course experiment using one of the GST:GUS lines (line 6) plants was carried out. Uninduced leaf samples were taken at time = 0 hours and with 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone safener was applied to 6 week old plants via root drenching. Leaf tissue was taken at time intervals thereafter and fluorometric GUS assays were carried out. Figure 2 shows GST:GUS expression peaks at 72 hours and that expression drops off after 5-7 days. Tissues were taken from the plants and fluorometric assays were carried out (see Figure 3A). Histochemical staining with X-gluc was carried out with tissue pieces from plants used in the fluorometric assay. Staining showed a constitutive GST:GUS expression in roots and stems; fluorometric results show a high uninduced GST:GUS expression which increases upon safener application. No expression could be seen in stolons from blue staining although fluorometric results show a high uninduced expression, increasing after chemical application (see Figure 3B). It can be seen from Figure 3C that constitutive expression of GST:GUS is mainly in the vascular area of the stained stem sections and from Figure 3D that staining of longitudinal sections through stem of safener induced plant stained with X-gluc that staining occurs in vascular tissue (xylem, cambium, phloem) at each side of the sectioned stem. A transverse section through uninduced 6 week old potato stem stained with X-gluc (see Figure 4) shows xylem (inner), cambium and phloem (outer) to be stained blue, indicating high GST:GUS expression in the vascular bundles in stems. Expression of GST:GUS in vascular tissue of the stem is constitutive.

Example 4

Four month old lines of G44 and G6, wild type and 35S:GUS were root drenched with 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone safener. Tubers from these lines were histochemically stained with X-gluc pre and post chemical treatment (see Figure 5A). Negative control wild type tubers show no staining as expected; positive control CaMV 35S tubers stained deep blue. G44 showed slight coloration in the centre of uninduced tubers whereas G6 tubers stained deep blue in uninduced and induced tubers showing constitutive expression of GST:GUS in tubers.

Example 5

A fluorometric assay of tubers from uninduced and induced plants as carried out (see Figure 5B). No expression from wild tpe plants was seen and a relatively high expression from CaMV 35S tubers was seen. Tubers from lines G6 and G44 have a high uninduced GUS expression which increases after safener application in G6 but decreases after treatment in line G44 tubers. Histochemical and fluorometric results point to constitutive expression of GST:GUS in tubers, with a G6 being the highest expressing line.

Example 6

Six month old tubers from lines from storage, G6 and G44, and a wild type negative control were histochemically stained. G6 and G44 stained well with X-gluc. G6 stained a deeper blue than G44 as can be seen from Figure 6A suggesting a higher expression of GST:GUS. This indicates that the expression of GST:GUS carries on through dormancy and is still on during storage at 6 months old.

Example 7

Eight month old tubers which were at the sprouting stage were histochemically stained. As can be seen from Figure 6B, G6 shows deep blue coloration with staining and shows GST is expressed throughout dormancy and throughout sprouting and does not appear to drop in expression levels.

Figures 7A and 7B show stained tubers throughout the potato life-cycle at 2, 4, and 8 months compared with the wild type and CaMV 35S controls. It can be clearly seen that the expression of GST:GUS remains high throughout dormancy and onto the new sprouting stage to be used as seed.

Example 8

The results showed that the GST -27 promoter region was inducible in leaves but expressed in the absence of inducer in roots, stems and tubers. The constitutive tuber expression was present in developing tubers, dormant tubers and sprouting tubers. Although constitutive expression of the GST-II-27 promoter in root tissue has already been described, expression of the GST-II-27 promoter in the stem and tuber organs was surprising.

Example 9

Comparison of GST-II-27:GUS and 35S:GUS transgenic plants.

Comparison of the full length 3822 base pair promoter to the patatin tuber-specific promoter, 35S promoter and to wild type tubers is carried out by analysing GUS expression.

Inducible leaf expression by application of chemical safener, 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone to various plant lines including GST-27:GUS (lines G6, G44 described above), CaMV 35S:GUS, patatin:GUS and wild type plants is analysed by fluorometric assay.

Uninduced tuber gene expression of the above lines is measured fluorimetrically and histochemically comparing GUS expression levels at all stages throughout dormancy. This allows a direct comparison between patatin, full GST-27 and deleted GST-27 promoter expression in tubers. There is some evidence that the level of expression of the patatin promoter decreases in tubers during dormancy; a promoter which is expressed at consistent high levels throughout the tuber dormancy and sprouting could be used to drive the expression of target genes in the suppression of sprouting of the tuber. The expression of the GST-27 promoter is determined throughout dormancy by comparison with the patatin promoter.

A deleted version of the GST-II-27 promoter region (SEQ ID NO: 2) was also prepared and tested (see Figure 11). This promoter region can then be compared with the 3822 base pair full length GST-II-27 promoter region.

It is envisaged that the present invention may also be useful in the expression of antibodies and storage proteins. The potato tuber is an important food source and it could therefore be used to express genes which are involved in the synthesis of micronutrients, for example, enzymes leading to expression of carotenoid or vitamin E. It is envisaged that the present invention could also be used to express genes which affect plastid number or size which could lead to an altered or increased starch content or other plastid component deposition.

Other modifications to the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

Example 10

Tubers with the deleted version (SEQ ID NO: 2) of the GST-II-27 promoter were harvested from plants at 18 weeks and stored at 4°C. At 22 weeks the tubers were sliced and histochemically stained with X-gluc to test for GUS expression (see Figure 12). The stained tubers show GST drives expression of GUS in the tuber without application of the chemical safener 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone and is constitutively expressed in tubers.

Example 11

A number of GST-II-27:GUS lines containing the full length 3.8 kb GST promoter were grown and induced whilst still attached to the plants at 15 weeks. Uninduced tubers were sampled from the plants before application of the chemical safener 3-dichloroacetyl-2,2,5-trimethyl-1,3-oxazolidone. Safener was applied at the rate of 20mg/40ml (0.5 g/l). Tuber samples were also harvested after safener application. The tubers were sliced and stained with X-gluc to detect GUS activity. Figure 13 shows the 3.8 kb GST-II-27:GUS lines after staining. The uninduced lines are stained blue, showing GUS activity before application of chemical inducer. The induced lines are also stained blue and have comparable GUS activity to the uninduced lines. This shows that a number of other lines containing the GST-II-27 promoter have constitutive expression in the tubers and it is not unique to lines GST-II-27:GUS 6 and GST-II-27:GUS 44.

Example 12

Analysis of expression of the GUS reporter gene under control of promoters GST-II-27, Patatin and CaMV 35S throughout the tuber life cycle.

In the Experiment described below, the following buffers were used.

Extraction Buffer for GUS Assays
0.1 M Na PO_4 (pH 7)
5 mM β-mercaptoethanol
10 mM Na_2EDTA
0.1% Triton X-100
H_2O to final volume

Assay Buffer for GUS Assays
1 mM 4-methyl umbelliferyl β-D glucuronide
80% extraction buffer
20% methanol

Staining Buffer
0.1 M monobasic/dibasic $NaHPO_4$ buffer (pH 7)
0.05% Triton X-100
0.1% Dimethyl sulphoxide
1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

The following lines of tissue culture plants were transferred into the glasshouse on 22/07/98:

GST-II-27:GUS 6 c.v. Solara (3.8 kb promoter)
GST-II-27:GUS 44 c.v. Solara (3.8 kb promoter)
842-03 c.v. Kardal (842 = Patatin:GUS)
842-06 c.v. Kardal
842-07 c.v. Kardal
410-89 c.v. Kardal (410 = CaMV 35S:GUS)
410-92 c.v. Kardal
410-95 c.v. Kardal
wild type c.v. Solara

Two plants per line were transferred from tissue culture to the glasshouse at the three node stage. For the first four weeks the plants were grown up in 3 inch pots in a mixture of 50% Sinclairs potting and bedding compost and 50% John Innes No. 3. During this time they were grown in a growth cabinet with an 18°C, 65% humidity, 12 hour day and a 12°C, 75% humidity, 12 hour night. The plants were fed until flowering with 3:0:1 (Nitrogen:Phosphorous:Potassium) liquid feed once a week and 1:0:2 ratio liquid feed twice a week. After the plants were established they were moved to a glasshouse bay with conditions of 18°C, 50% humidity, 16 hour day and 14°C, 70% humidity, 8 hour night. The light levels in the glasshouse averaged at 43 klux.

Tubers were harvested when the plants showed signs of senescence; at this point the plants were in soil for 18 weeks. The tubers were washed in water, dried and placed in nylon bags. The bags were originally stored in a seed store at 13°C, 30% humidity in the dark for 5 weeks; the stored tubers were moved from this store at 23 weeks into a cooler store at 4°C, 30% humidity.

Tuber samples were taken from the plants at regular intervals throughout the tuber life cycle, as indicated below.

Sampling times: Week	Date	Developmental Stage
0	22/07/98	Tissue culture plants transferred to glasshouse
8	23/09/98	Growing plants
18	01/12/98	At harvest
23	05/01/98	In storage
35	29/03/98	Eyes formed
47	24/06/98	Sprouting tubers

At each time point, two tubers were sampled per line (one tuber per plant). Two bores were taken from each tuber using a metal borer, 0.5cm wide and 1.5 cm-2 cm in length. The tuber bores were stored in GUS extraction buffer at -80°C until all samples were collected. Tuber slices were also cut from the sampled tubers at each time point and histochemically stained using X-gluc, which gave an initial measure of GUS activity.

When the final tuber samples were collected after sprouting, all bores from each time point were removed from -80°C. The samples were ground, centrifuged and GUS assays were carried out on the supernatant using 4-methyl umbelliferyl β-D glucuronide (MUG) to measure fluorometrically the level of GUS expression through dormancy in all lines. GUS activity and protein levels were measured from each sample. The lines GST-II-27:GUS 6 and GST-II-27:GUS 44 were compared to high GUS expressing lines Patatin:GUS and 35S:GUS.

The results of the experiments are shown in Figures 14 to 16.

Conclusions:

All lines showed blue coloration after staining with X-gluc, although this varied in intensity from line to line (see photograph). This showed every line analysed expressed GUS.

GST-II-27:GUS 6 is expressed in tuber tissue throughout dormancy and breakage of dormancy in the absence of a chemical inducer (Figures14, 15, 16b). A slight fluctuation is seen in the expression level of GST-II-27:GUS 6 in growing tubers (week 8), (Figure 16b). Overall, GST-II-27 expresses at an even level throughout the life cycle of the tuber.

Patatin expression shows a general decrease of expression throughout the tuber life cycle (Figures 14, 16c, 16d, 16e).

Patatin:GUS line 842-07 is the lowest expressing patatin line tested here and has lower GUS expression than GST-II-27:GUS 6 after dormancy break (Figure 16e).

The Patatin:GUS high expressing lines analysed here have a greater level of expression than the GST-II-27:GUS lines analysed (e.g. 842-06) (Figure 14).

842-03 and 842-06 Patatin:GUS lines have a higher level of expression than the CaMV 35S:GUS lines tested in tubers (410-89, 410-92, 410-95) (Figure 15).

The GST-II-27 promoter is expressed constitutively throughout potato tuber development, dormancy and dormancy breakage. The GST-II-27 lines used here express GUS at a lower level than the Patatin promoter lines analysed; however, it can be seen that the level of expression is more uniform in the GST-II-27 lines.

Example 13

GST tuber lines were compared throughout the tuber life cycle to Patatin:GUS (842), 35S:GUS (410) and wild type tuber lines. Tubers were taken from the plants at regular intervals and sampled during storage. Tubers were sliced and histochemically stained with X-gluc. Figure 17 shows the stained tubers at all stages during the tuber development. It is clearly shown that GST-II-27:GUS 6 is expressed constitutively throughout endodormancy, ecodormancy and paradormancy at uniform levels. GST-II-27:GUS 44 is expressed throughout the tuber life cycle at a lower level than 35S:GUS and Patatin:GUS lines.

Example 14

Figure 18 shows the size of the sprouts on the tubers of lines GST-II-27:GUS 6, GST-II-27:GUS 44, 35S:GUS (410), Patatin:GUS (842) and wild type. The tubers were removed from storage at 47 weeks and contained sprouts of between 0.5 and 3cm in length. The tubers were sampled for histochemical (see Figure 17) and fluorometric GUS analysis.

Example 15

GST-II-27:GUS line 6 tuber was sampled at 47 weeks. The tuber was sprouting and was sliced and histochemically stained using X-gluc. The tuber shows expression of GUS activity at sprouting (Figure 19).

CLAIMS

1. A method of constitutively expressing a target gene in a storage organ or stem of a plant comprising incorporating, preferably stably incorporating, into the genome of the plant a DNA construct comprising a first polynucleotide sequence comprising the gene promoter region for the 27 kD subunit of the glutathione-S-transferase, isoform II, or a deleted fragment thereof which retains the activity of the gene promoter region, operably linked to and controlling a target gene sequence.

2. A method according to claim 1 wherein the DNA construct further comprises a second polynucleotide sequence comprising at least one DNA sequence operably linked to and under the control of an inducible promoter region.

3. A method of constitutively expressing a target gene in a storage organ or stem of a plant comprising incorporating, preferably stably incorporating, into the genome of the plant a DNA construct comprising a first polynucleotide sequence comprising the gene promoter region for the 27 kD subunit of the glutathione-S-transferase, isoform II, or a deleted fragment thereof which retains the activity of the gene promoter region, operably linked to and controlling a target gene sequence, the DNA construct further comprising a second polynucleotide sequence comprising at least one DNA sequence operably linked to and under the control of an inducible promoter region.

4. A method according to any one of claims 1 to 3 wherein expression of the target gene results in the suppression of sprouting of the storage organ.

5. A method according to any one of claims 2 to 4 wherein the suppression of sprouting is neutralised by inducing expression of the second polynucleotide sequence.

6. A method according to any one of claims 2 to 5 wherein the second polynucleotide sequence is a sense, antisense or partial sense sequence corresponding to said first polynucleotide sequence or a DNA sequence capable of causing suppression of the

protein encoded by the target gene.

7. A method according to any one of the preceding claims wherein the first and second polynucleotide sequences further comprise a transcription terminator region.

8. A method according to any one of the preceding claims wherein the gene promoter is the maize 27 kD subunit of the glutathione-S-transferase, isoform II gene promoter (SEQ ID NO: 1) or a sequence substantially homologous thereto.

9. A method according to any one of claims 1 to 8, where the gene promoter is a fragment of the 27 kD subunit of the glutathione-S-transferase, isoform II gene promoter which retains the activity of the gene promoter region.

10. A method according to claim 9, wherein the fragment is one of the following regions of the 27 kD subunit of glutathione-S-transferase:
a sequence comprising 897 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase;
a sequence comprising 570 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase;
a sequence comprising 378 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase;
or the 693 base fragment of SEQ ID NO: 2.

11. A method according to any one of the preceding claims wherein the storage organ is a tuber.

12. A method according to any one of the preceding claims wherein the plant is a potato plant.

13. A method according to any one of the preceding claims wherein the target DNA sequence codes a protein selected from the group consisting of

proteins involved in the mobilisation of reserves during dormancy e.g.starch breakdown, i.e. starch phosphorylase, amylase (e.g. α or β amylase) and maltase; e.g. in glycolysis and subsequent metabolism e.g. phosphofructokinase, hexokinase; proteins involved in in sucrose biosynthesis e.g. sucrose synthase; proteins involved in the transport of reserves during dormancy such as in phloem loading e.g. ATPase; proteins involved in long distance phloem transport and in phloem unloading e.g.inorganic pyrophosphorylase (iPPase); and proteins involved in the utilisation of reserves during dormancy such as in assimilate breakdown e.g. the breakdown of sucrose in the growing sprout, i.e. invertase; and in the utilisation of assimilates e.g. utilisation of sucrose-derived metabolites, in the provision of energy required for sprout formation e.g.adenine nucleotide translocator (ANT) and malate oxoglutarate translocator (MOT) and inhibitors thereof such as uncoupling proteins; or a sequence which is involved in potato sprouting.

14. A method according to claim 13 wherein the target DNA sequence is one of those resulting in the production of sense, anti-sense or partial sense sequence(s) to, and/or coding for, proteins involved in the mobilisation and/or utilisation of sucrose, in potato sprouting and in mitochondrial function, such as in respiration.

15. A method according to claim 14 wherein the target DNA sequence is one of those coding for an invertase derived from plant, bacterial or fungal sources e.g. from yeast, a pyrophosphatase derived from plant, bacterial or fungal sources and proteins involved in mitochondrial function such as adenosine nucleotide translocator protein (ANT) or mitochondrial oxoglutarate translocator (MOT) derived from plant, bacterial or fungal sources.

16. A DNA construct comprising comprising the gene promoter region for the 27 kD subunit of the glutathione-S-transferase, isoform II, or a deleted fragment thereof which retains the activity of the gene promoter region, operably linked to and

controlling a target gene sequence as defined in any one of claims 4 or 13 to 15.

17. A DNA construct as claimed in claim 16 wherein the gene promoter is the maize 27 kD subunit of the glutathione-S-transferase, isoform II gene promoter (SEQ ID NO: 1) or a sequence substantially homologous thereto.

18. A DNA construct according to claim 16 wherein the fragment is one of the following regions of the 27 kD subunit of glutathione-S-transferase:
a sequence comprising 897 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase;
a sequence comprising 570 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase;
a sequence comprising 378 base pairs immediately upstream of the transcription start point of the gene promoter sequence of the 27kD subunit of glutathione S-transferase;
or the 693 base fragment of SEQ ID NO: 2.

19. A DNA construct according to any one of claims 16 to 18 further comprising a second polynucleotide sequence comprising at least one DNA sequence operably linked to and under the control of an inducible promoter region.

20. A DNA construct according to claim 19 wherein the second polynucleotide sequence is a sense, antisense or partial sense sequence corresponding to said first polynucleotide sequence or a DNA sequence capable of causing suppression of the protein encoded by the target gene.

21. A DNA construct according to any one of claims 16 to 20 wherein the first and second polynucleotide sequences further comprise a transcription terminator region.

22. Potato plant germ plasm comprising a construct as claimed in any one of claims 16 to 21.

23. A potato plant, potato seed or potato plant cell comprising a DNA construct as claimed in any one of claims 16 to 22.

24. A method for preventing or inhibiting sprouting in a potato tuber, the method comprising causing the tuber to express a target sequence as defined in any one of claims 4 or 13 to 15 under the control of the gene promoter region for the 27 kD subunit of the glutathione-S-transferase, isoform II, or a deleted fragment thereof which retains the activity of the gene promoter region.

Fig.1A.

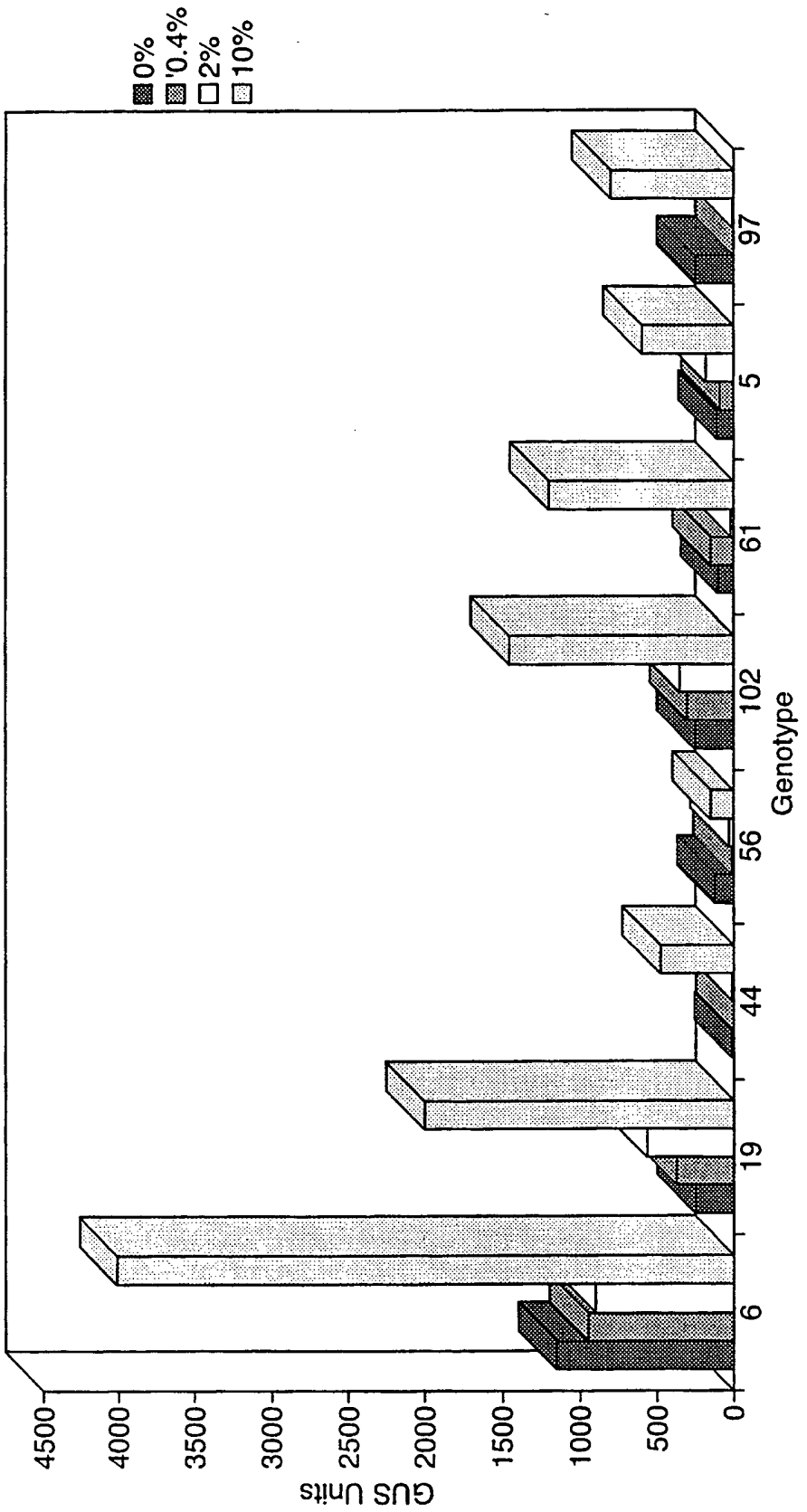

0%
0.4%
2%
10%
GUS Units
4500
4000
3500
3000
2500
2000
1500
1000
500
0
6
19
44
56
102
61
5
97
Genotype

Fig.1B.

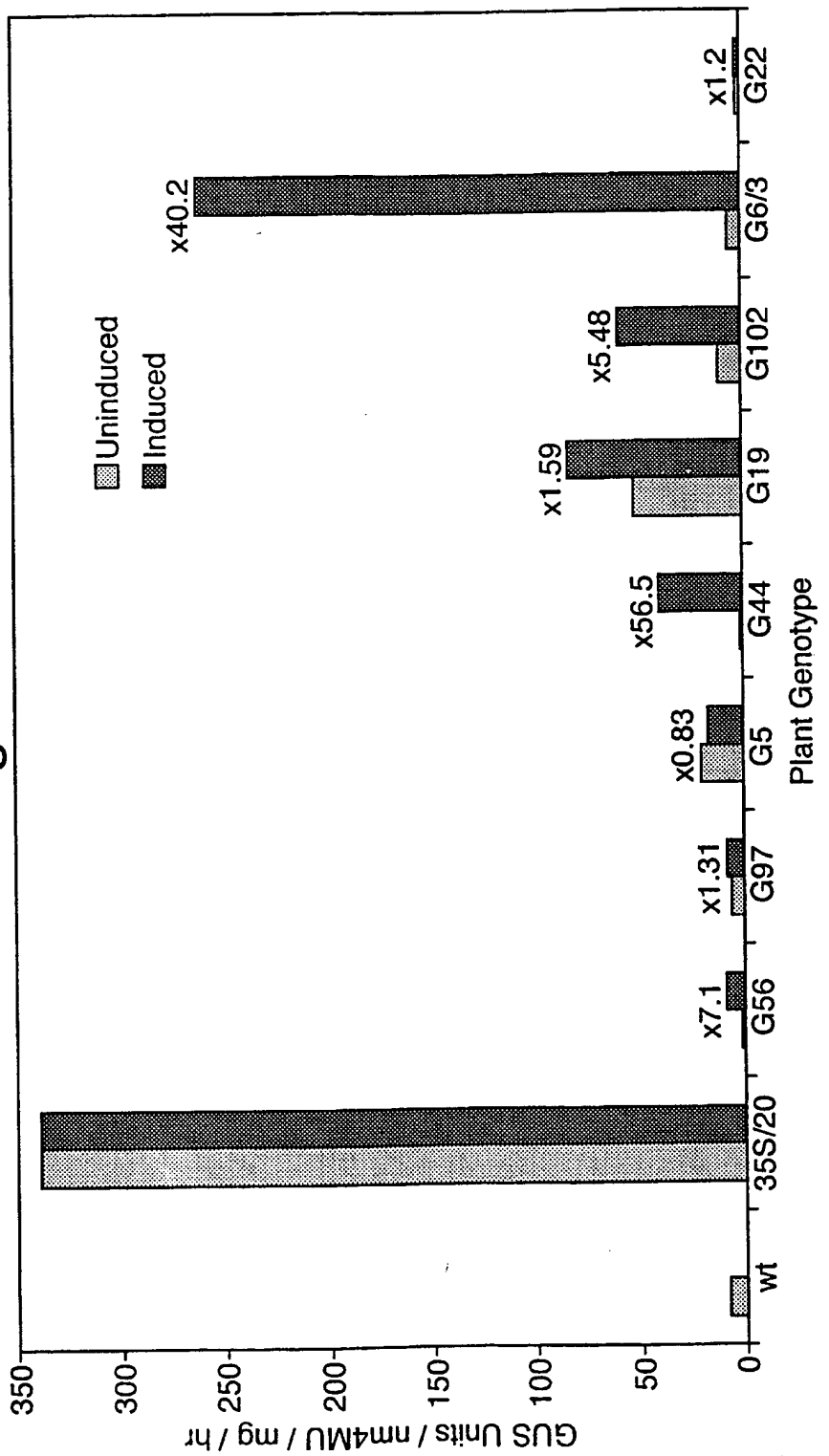

GUS Units / nm4MU / mg / hr
350
300
250
200
150
100
50
0
Uninduced
Induced
x40.2
x5.48
x1.59
x56.5
x0.83
x1.31
x7.1
x1.2
wt
35S/20
G56
G97
G5
G44
G19
G102
G6/3
G22
Plant Genotype

Fig.2.

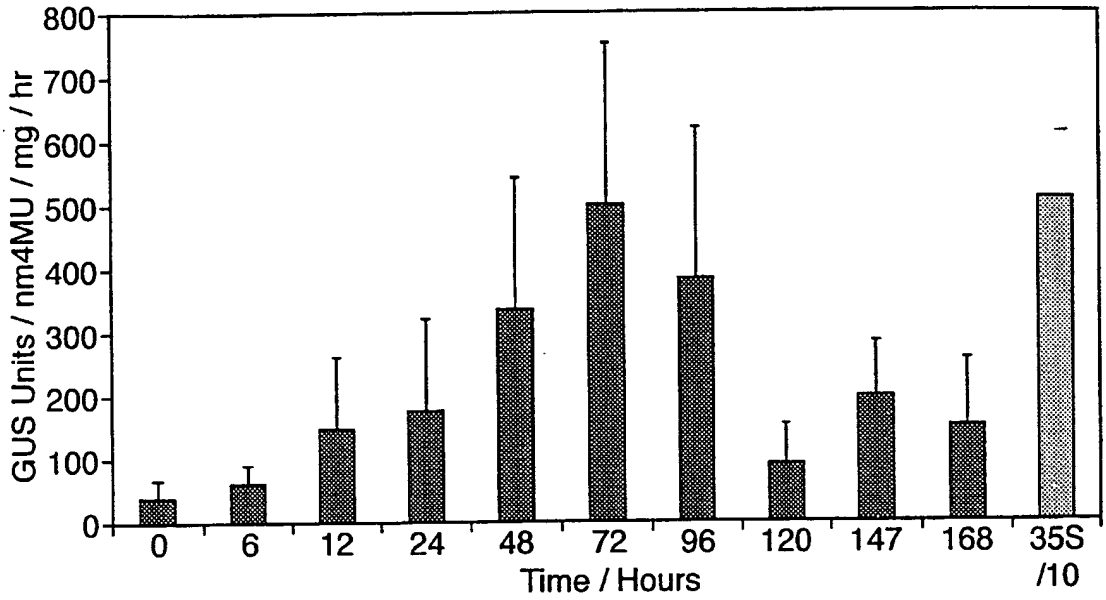

800
700
600
500
400
300
200
100
0
GUS Units / nm4MU / mg / hr
0
6
12
24
48
72
96
120
147
168
35S /10
Time / Hours

Fig.3A.

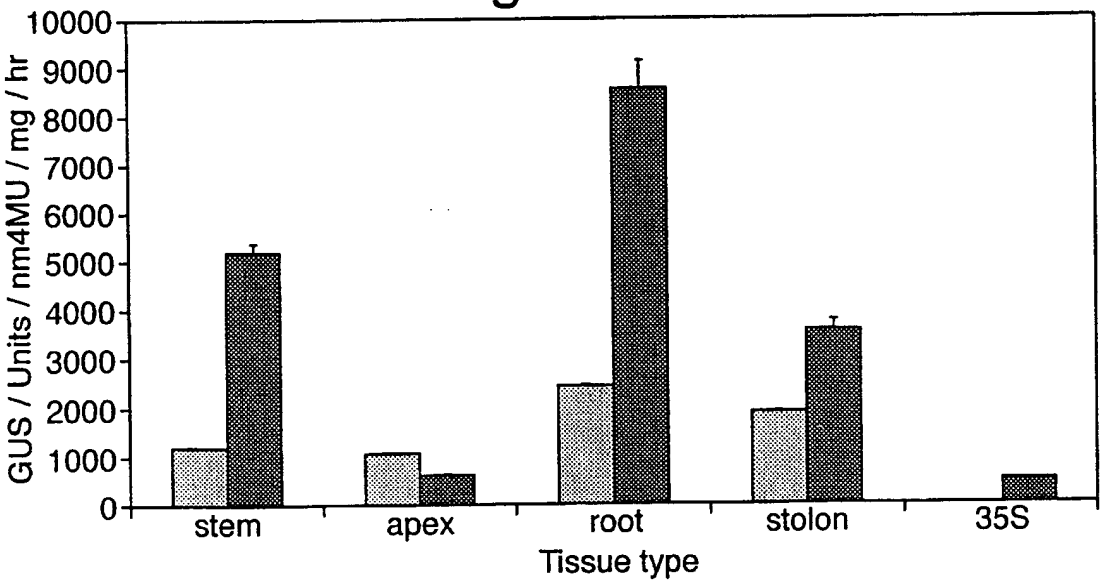

10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
GUS / Units / nm4MU / mg / hr
stem
apex
root
stolon
35S
Tissue type

Fig.3B.

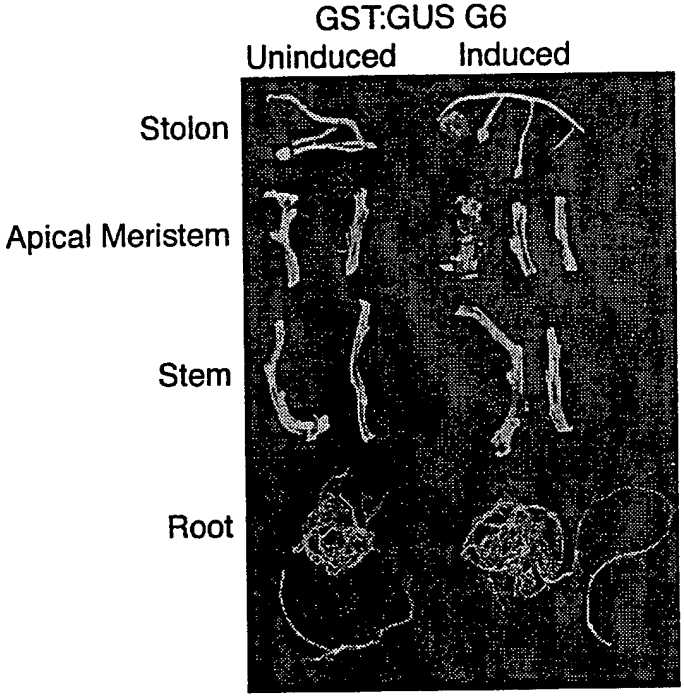

GST:GUS G6
Uninduced
Induced
Stolon
Apical Meristem
Stem
Root

Fig.3C.

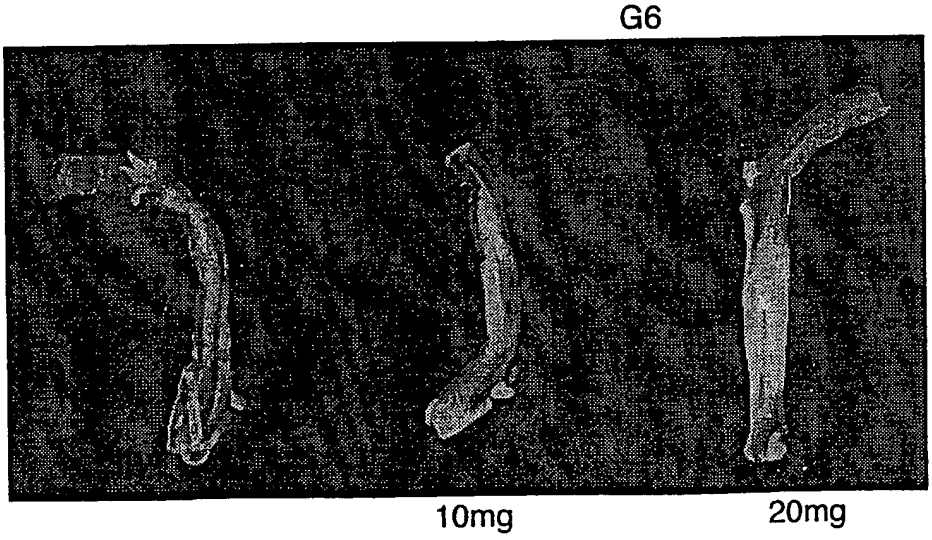

G6
10mg
20mg

Fig.3D.

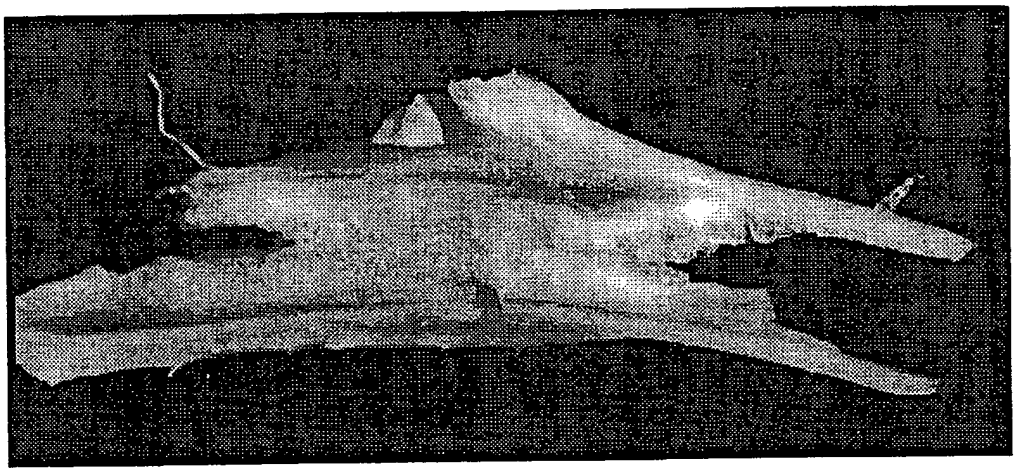


Fig.4.

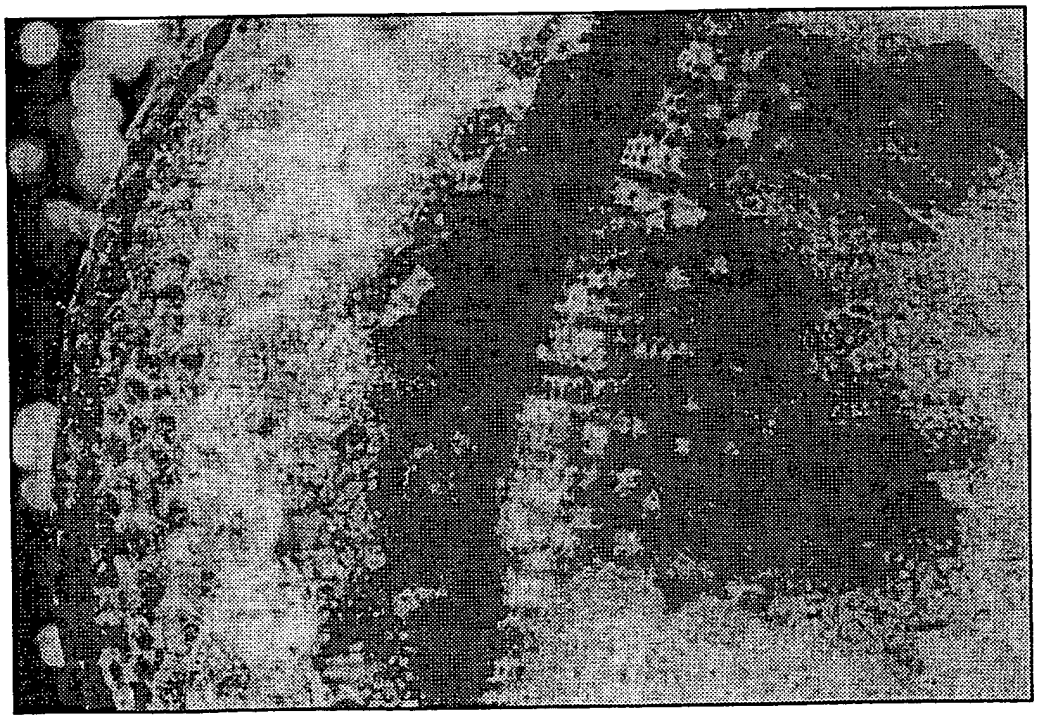


Fig.5A.

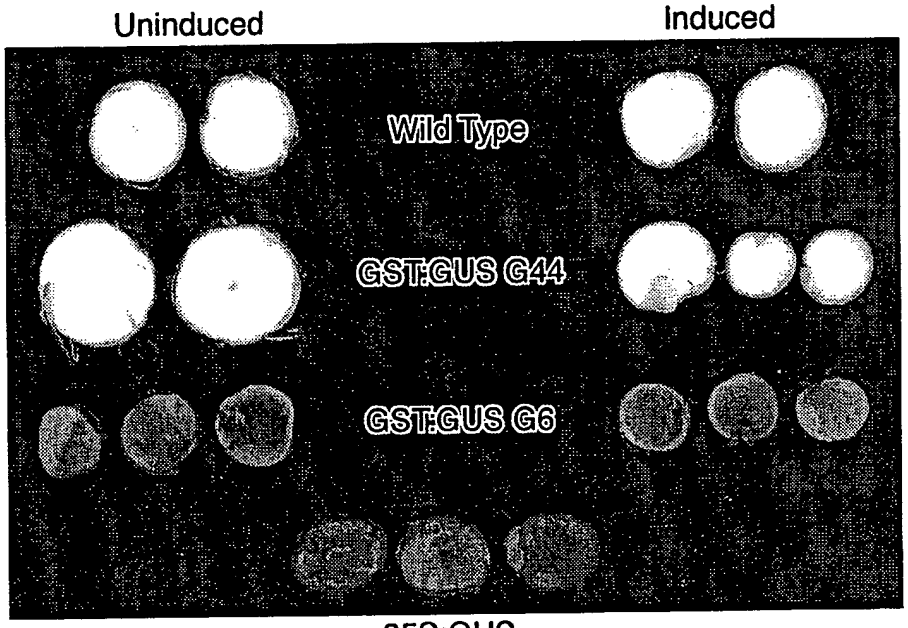

Uninduced
Induced
Wild Type
GST:GUS G44
GST:GUS G6
35S:GUS

Fig.5B.

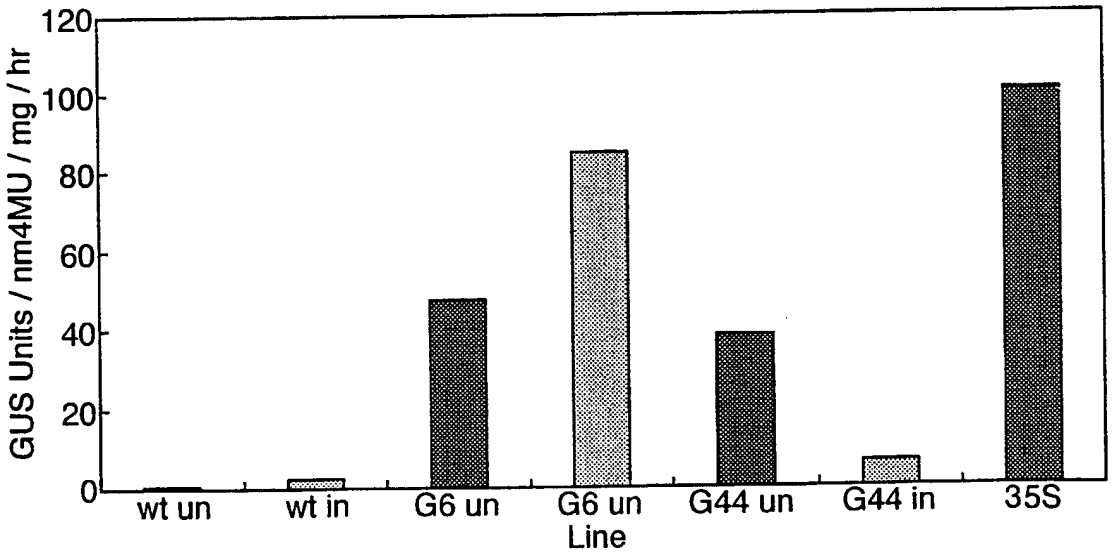
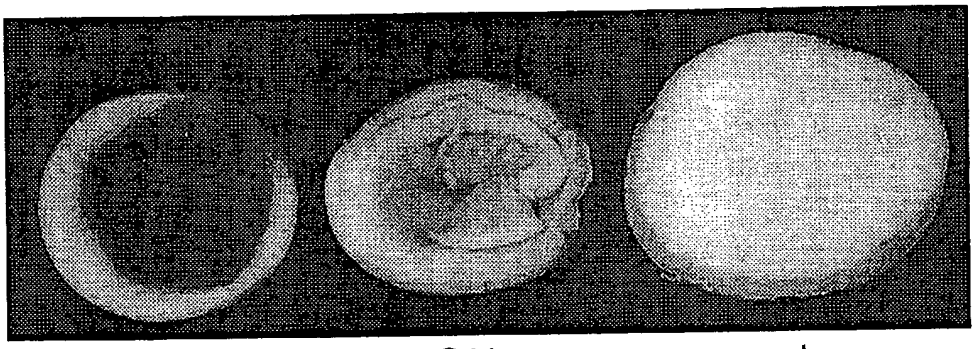

GUS Units / nm4MU / mg / hr
120
100
80
60
40
20
0
wt un
wt in
G6 un
G6 un
G44 un
G44 in
35S
Line

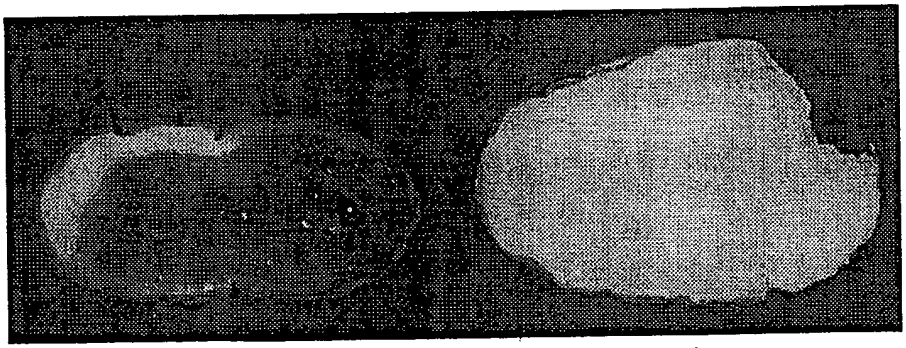
Fig.6A.



G6 G44 wt

Stored

Fig.6B.



G6 wt

Sprouting

Fig.7A.

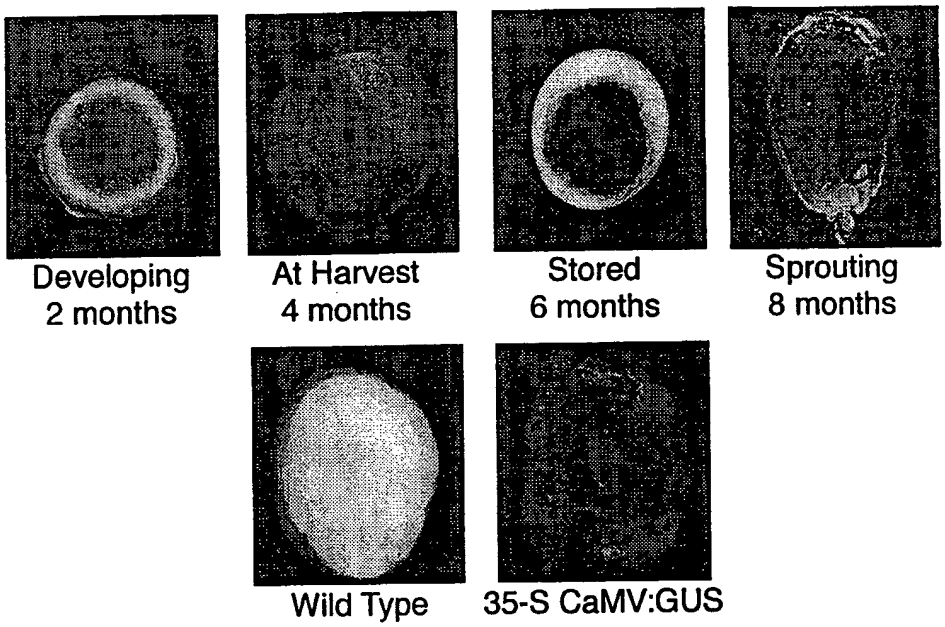
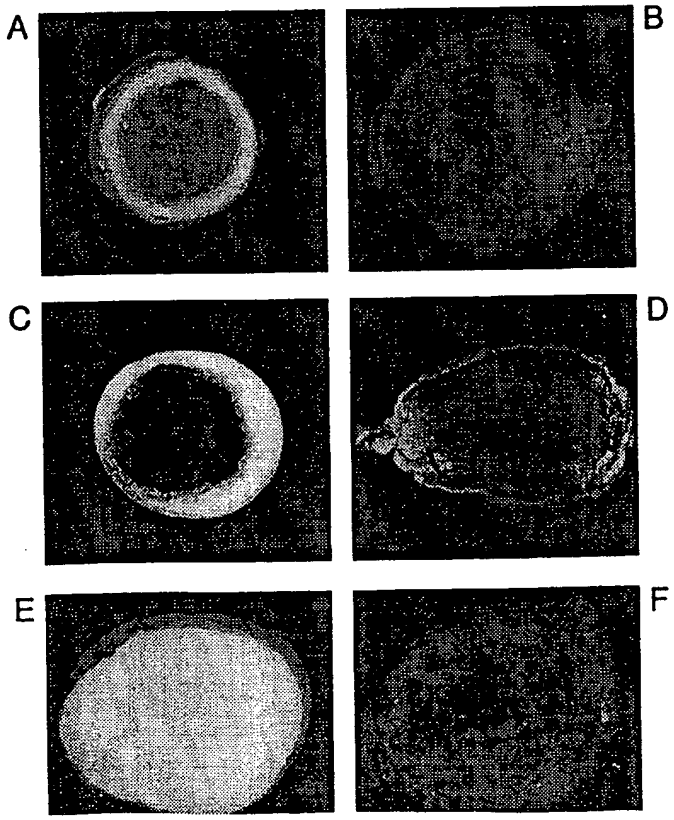

Developing
2 months
At Harvest
4 months
Stored
6 months
Sprouting
8 months
Wild Type
35-S CaMV:GUS

Fig.7B.


A
B
C
D
E
F

Full GST promoter: Fig.8.

aattccaaatatatgatgattgttgtcctagtgcagaagaactaaatatactagcgaaaaaaaaccttcctagtcatgtaagtgtatgggca
tatagaaaaataaacatctcaagactccaaactagtcatagcttttagtcacaacttcaaacacttcatgccaacaagatcatggatttttttt
tttgcctaagacaaaactagaatgagaaaagaactaactcatcatacatattagtatggcatcacaaaaaaaatgacacatatatgatact
atatcacacaggccttcagtttctagaacaagtgcagatcgatgtgtgggtatgcatgtctaatattttactaggttggatatgcatgggcg
ttcattcagaatcagtttcacacagtttatcgcacttctgtttacaaaacatggatttcattgctctgtactggctacatgcgtaaggatcaac
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gacaggaaactgatgtgcaacaacaaaaactaaaatagaaggaaacacaagttccaaatgtataataattgtcaccatagtgcaaaa
gaaccaaatatactgcagagaaaacttcctagtcatgtaagtgtatggacatatagaaataaaacatctcaagactccaataacaggctc
aagctaactagtcatggctttaaaccttcatgatgcaaactagtcacaactttaaacatttcatgccaacaagatcatggatggtgttttttttt
cctagggaaaagctagaatgagaaaagacctaactcagcatacatatcaggatagtatcgtatagacacgtatatgatactatatcacg
cagccgttcaatttctagaacaaatgcagattgatctgtgaatatgcatgtctcatattttactaggttggatggactgaatcccgtgaaac
aaacaatttattcaacaagtttctgcatgaatatcatctcaaattcaataatcactctcgttgataaaaaaaaatgcaaccaacagttaaccag
aagtgaaatagaaactatttgaatcagatcactccgttattcacatcaaaataattgttgcttgatctataaaagcagtaggaacattgtttac
ccatcaatttcaagtacacagtaacaagaacagtacagctagaattgagcatgtgagtattgttgatacctcgttgagctctctctgccgc
ggctttctgctcggcagcaagagccagctcaggatccaccccgaaagcttgggcgtaggtgttgtctatcggcgaaaacacgcgcgg
tacgccaagaacagcgcggccatctccatcccaggcacggtgcgcccgctttttcgccgtctcgctgagtcacggcgggcgtccagc
aggtagttgagcgccttccgcggcacgaatcgctgcgtgcggcccggatctggtcgagttggtagtcagcgtcggtgtcgaatgccg
ggacgtcgaccaggaagaagttgccgtcgctggggtggggacggaaggcgtcaggattgtcgcaagggcagagcccagcctgcg
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gtggtcctcgaaggggacgagctcgctggggtcaaaccaccccatagctcgagtcaccgaagaaggcgacgaggacgagcccgt
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cggggtcggacatcctgtggaggggaaccgaaaacctagatttggatgcaggttcgattggtctgggcttgggtttgggttccggagg
agggtggcctgggatcggtggaaggagggacattgttggtaatttttattattttataatatggagaaattcgagagactgaacgatggt
gatgtttatttgaggactatgtagtataaagtgtaaaatagtattttatcaagtttatattcacgtttttgctgaagatagtataatagtggagtt
gtttttggcggctacataatcttaggctatcttctcggtcgctctcatatcatatctactatcacattctctatttttaaatttcactttgtgtaatcta
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tatgatttataggcgattgggtagatgtgatttgataggtggttatgtaggagcgatttagtgagacattgtaaataattaggttgatgtgat
ccgaggatggctaggtagatatgattttaatggatggtttggtggactaagttatgtggacattataatatgttttaaatttctaagaaattgtt
tgtgttaaattgtatcccacatagattatttagccatctcaaagagaggtttgggttgtttacacaaataaaatattcgtttgcttctacaattta
tatgttttttatttacatgaaaactatatttttttattcatctactcacccagcacagaaattctggttgagtagatgaaaaaaaactacaacaaa
ctcttcctgaaagtgtcggtgtgaagccgagaaatccttttcatttcggtgacggagccccttgctggctgctgctcagtgcactccgttc
gcctgcctgccactacaagcgacggccgacgactcgcaagtatcggtaggcattttaaaactgaaaaccaaatctaaacccgaataga
ccaaattgttggtttattcgggtttttgggttcggattcggtttctaaatatgctatattttagggtataggttcg

5' Primer .....

ggttcagtttctaacctttaaaacctgaatagacgaataacccgaaatataaaaaatctcttaatatgtgatgatattattatatgatttatgaa
cttattaaccgaaaataatgataccatcctaacgatagtatatatatctatgtatgctatttttatagtcacttgttgtaataatagtacttccaat
taattaatcagtgtatatattttaacaaaagatactagcctctctactatttgagtatattcggtgcaccgaatagaccgaaccgaaattgtaa
gtctattcaggttcggttcctaaaattatttttaaaaattttggttctcatatttcagaatccgaaatttcataaatccaaatagaccgaaccaaa
ttacgctaatagaccgaataactagcgtactcgcaagtcgcaccccaCtagcctgctgcgtgcgtaagcgaggacgtcacgcgttctc
cctcccgtcgaccaaatacacttggtcttctagcaccttcttcctctccaaga

Fig.8 (Cont).

ctccaatcccccaaccaccagaaccagcgccagctctaacgtcacctctgatttctctctcctctctattgc

Pst I

.....

tagctgctttattataagtagcagctgcagcaggcaggagctgcacacacccatccaattccagctgctgatcttgatcctg

AvaI

.....

caccccgagccgtacacaagagctagtcggtagaacttgcaggagcggagcagaactaagtgcagagaacaggaca

Fig.9.

Deleted GST sequence

cttaatatgtgatgatattattatatgatttatgaacttattaaccgaaaataatgataccatcctaacgatagtatatatatctatgtatgctattt
ttatagtcacttgttgtaataatagtacttccaattaattaatcagtgtatatattttaacaaaagatactagcctctctactatttgagtatattcg
gtgcaccgaatagaccgaaccgaaattgtaagtctattcaggttcggttcctaaaattattttaaaaattttggttctcatatttcagaatccg
aaatttcataaatccaaatagaccgaaccaaattacgctaatagaccgaataactagcgtactcgcaagtcgcaccccactagcctgct
gcgtgcgtaagcgaggacgtcacgcgttctccctcccgtcgaccaaatacacttggtcttctagcaccttcttcctctccaagactccaat
cccccaaccaccagaaccagcgccagctctaacgtcacctctgatttctctctcctctctattgctagctgctttattataagtagcagctg
cagcaggcaggagctgcacacacccatccaattccagctgctgatcttgatcctgcaccccgagccgtacacaagagctagtcggta
gaacttgcaggagcggagcagaactaagtgcagagaacaggaca

Fig.10.

FULL GST PROMOTER (in Bin vector) as was transformed into plants

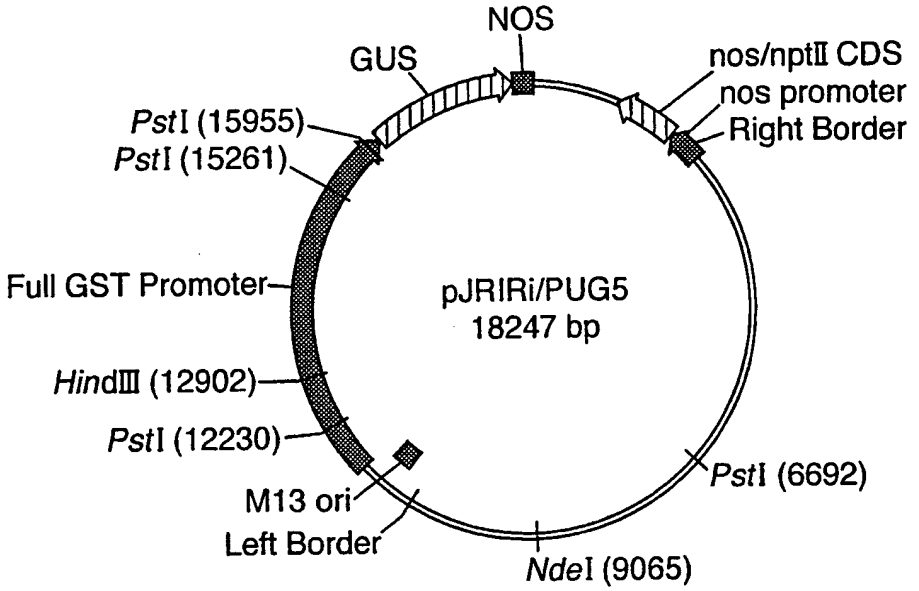
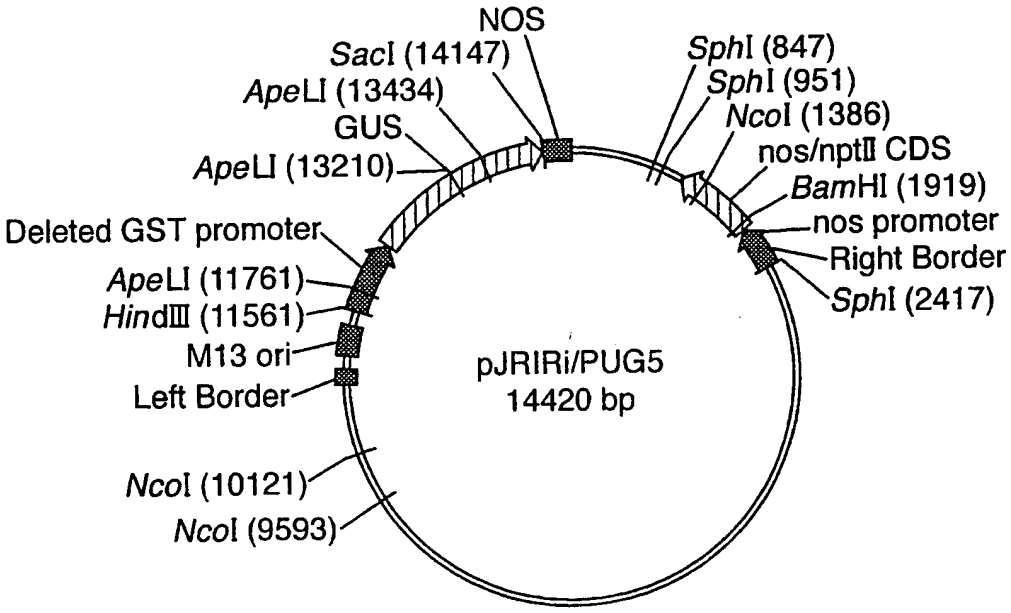
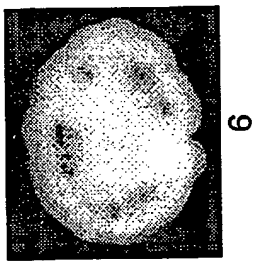
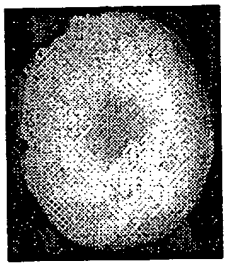


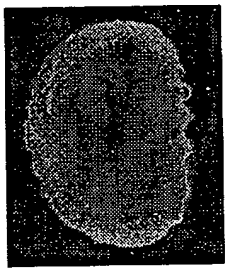
Fig.11.

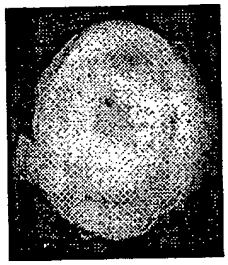
DELETED GST PROMOTER (570 bp promoter used in new transformations. In Bin vector as found transformed plants).

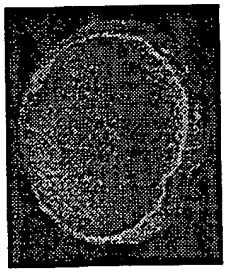












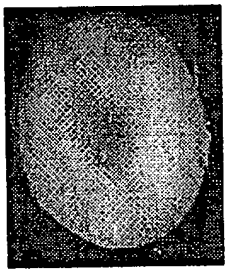
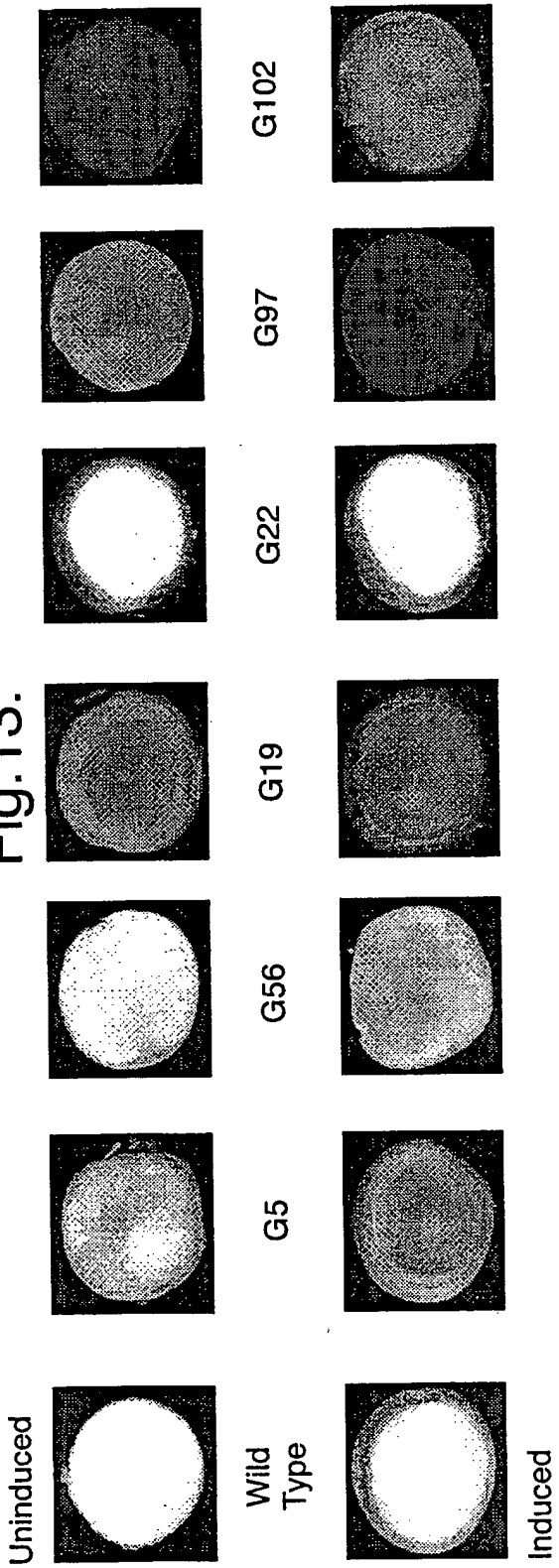


Fig.12.

Fig.13.



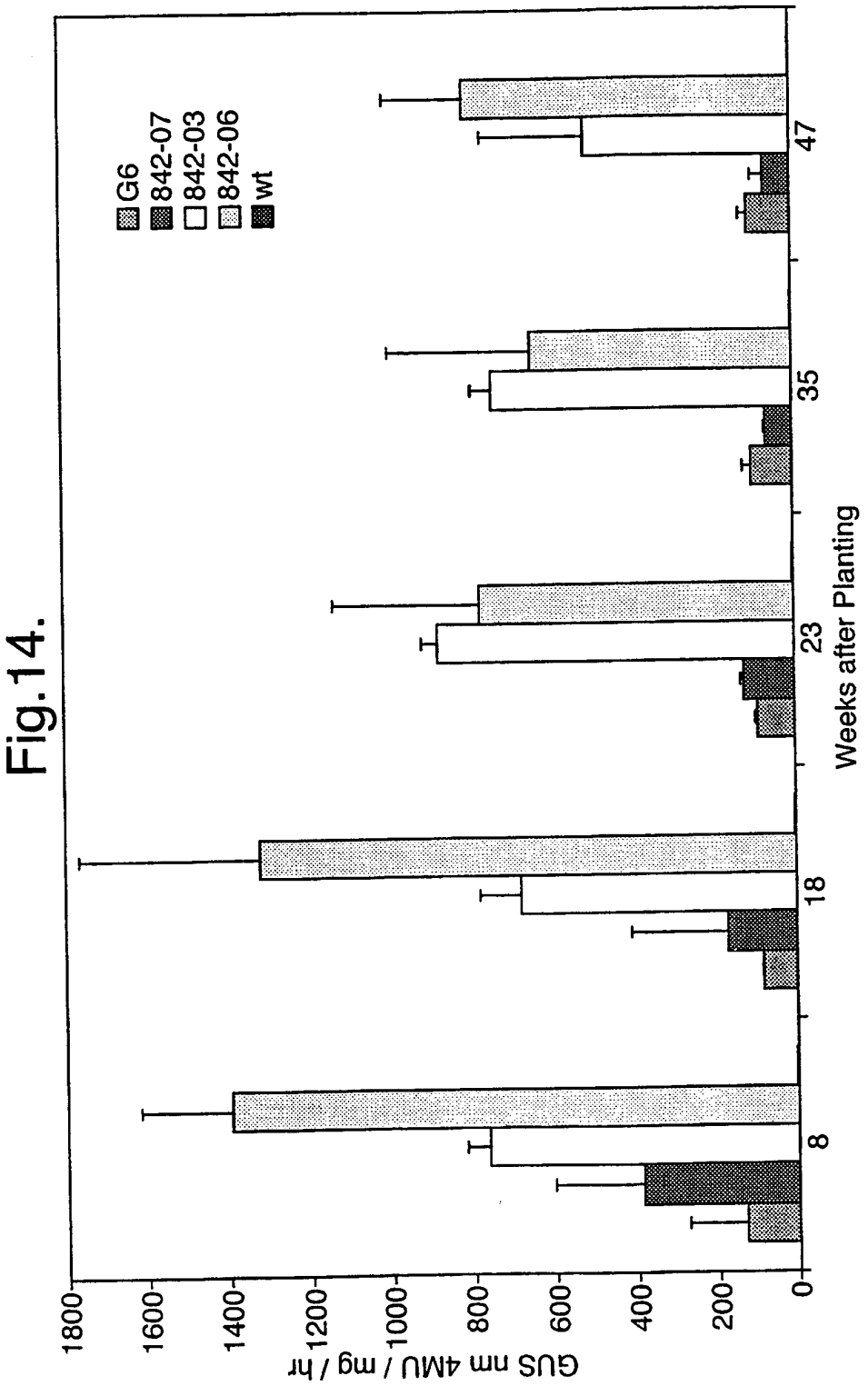

G6
842-07
842-03
842-06
wt
GUS nm 4MU / mg / hr
0
200
400
600
800
1000
1200
1400
1600
1800
8
18
23
35
47
Weeks after Planting

Fig.14.

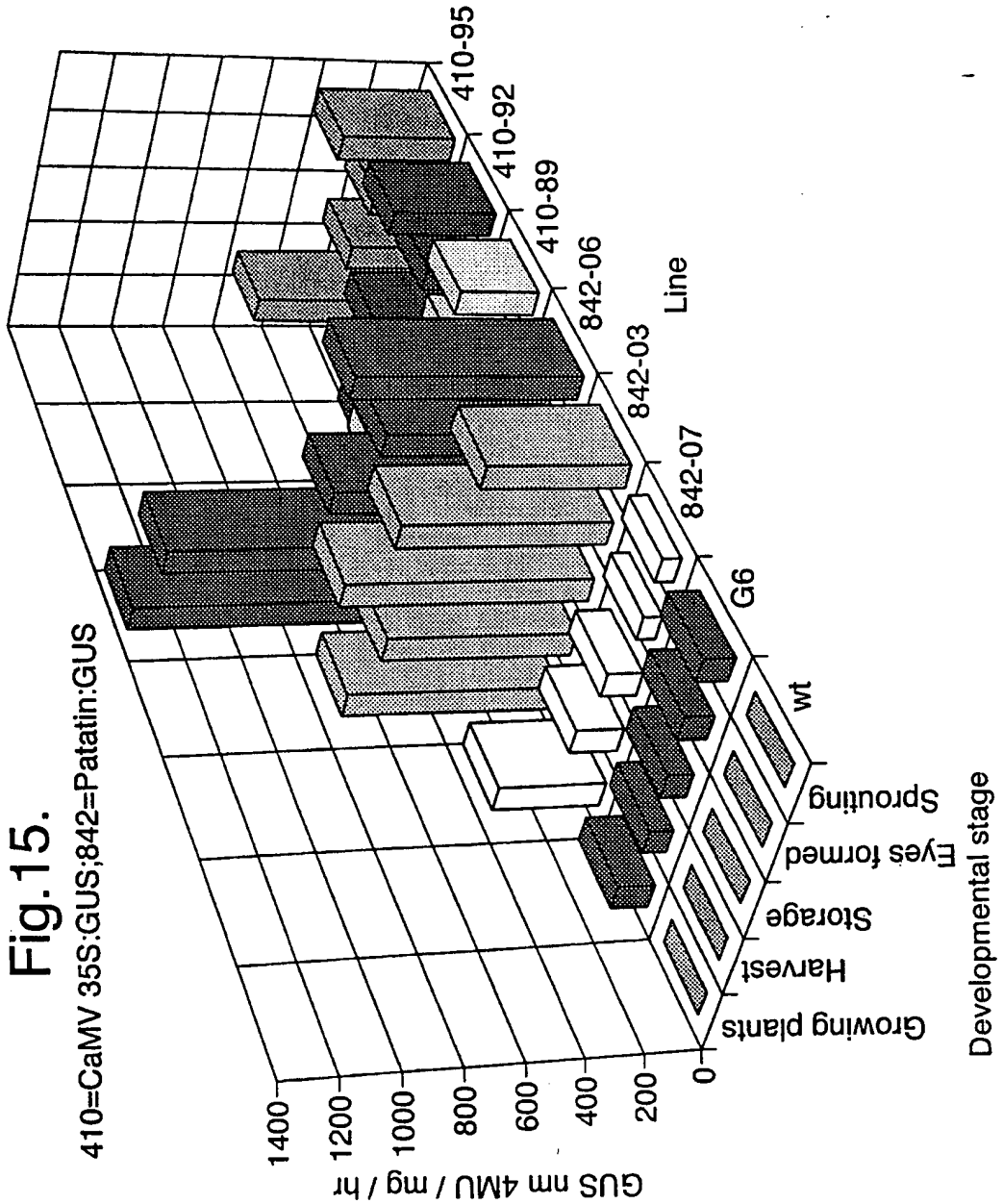

410=CaMV 35S:GUS;842=Patatin:GUS
GUS nm 4MU / mg / hr
0
200
400
600
800
1000
1200
1400
Developmental stage
Growing plants
Harvest
Storage
Eyes formed
Sprouting
wt
G6
842-07
842-03
842-06
410-89
410-92
410-95
Line

Fig.15.

Fig.16A.

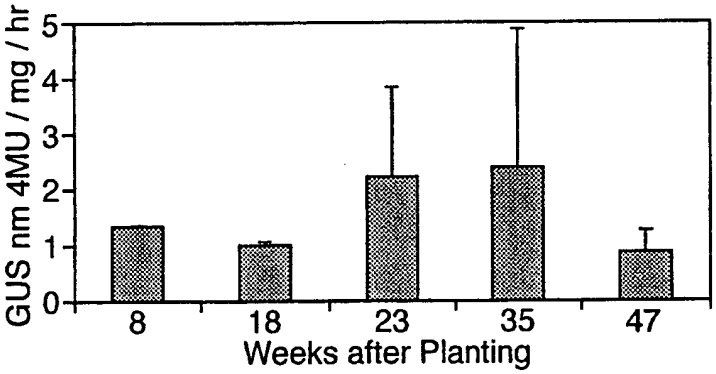


Fig.16B.

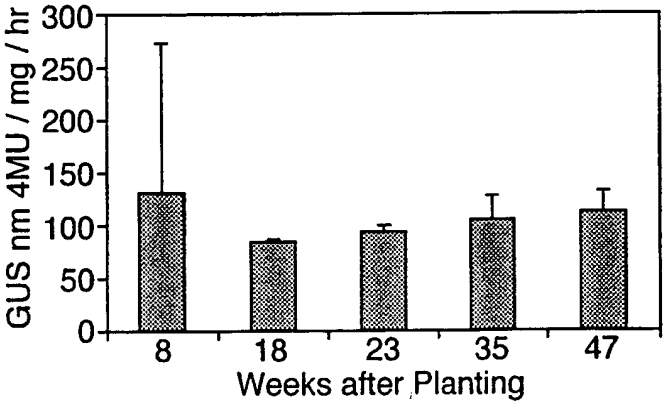


Fig.16C.

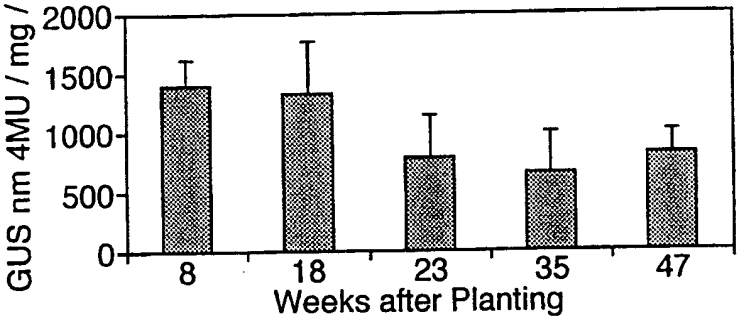


Fig.16D.

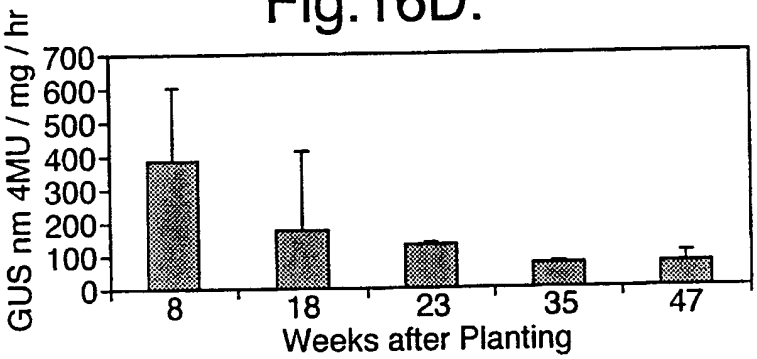
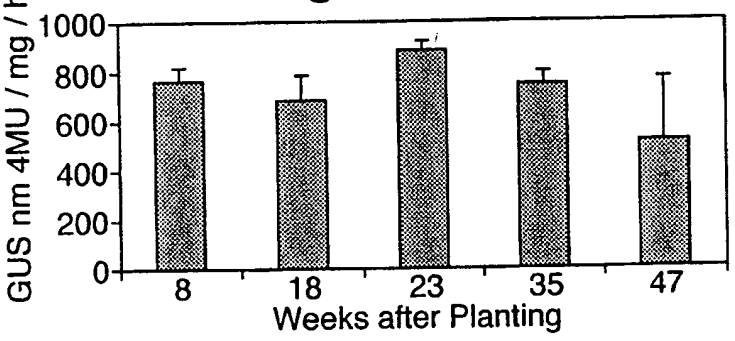


Fig.16E.



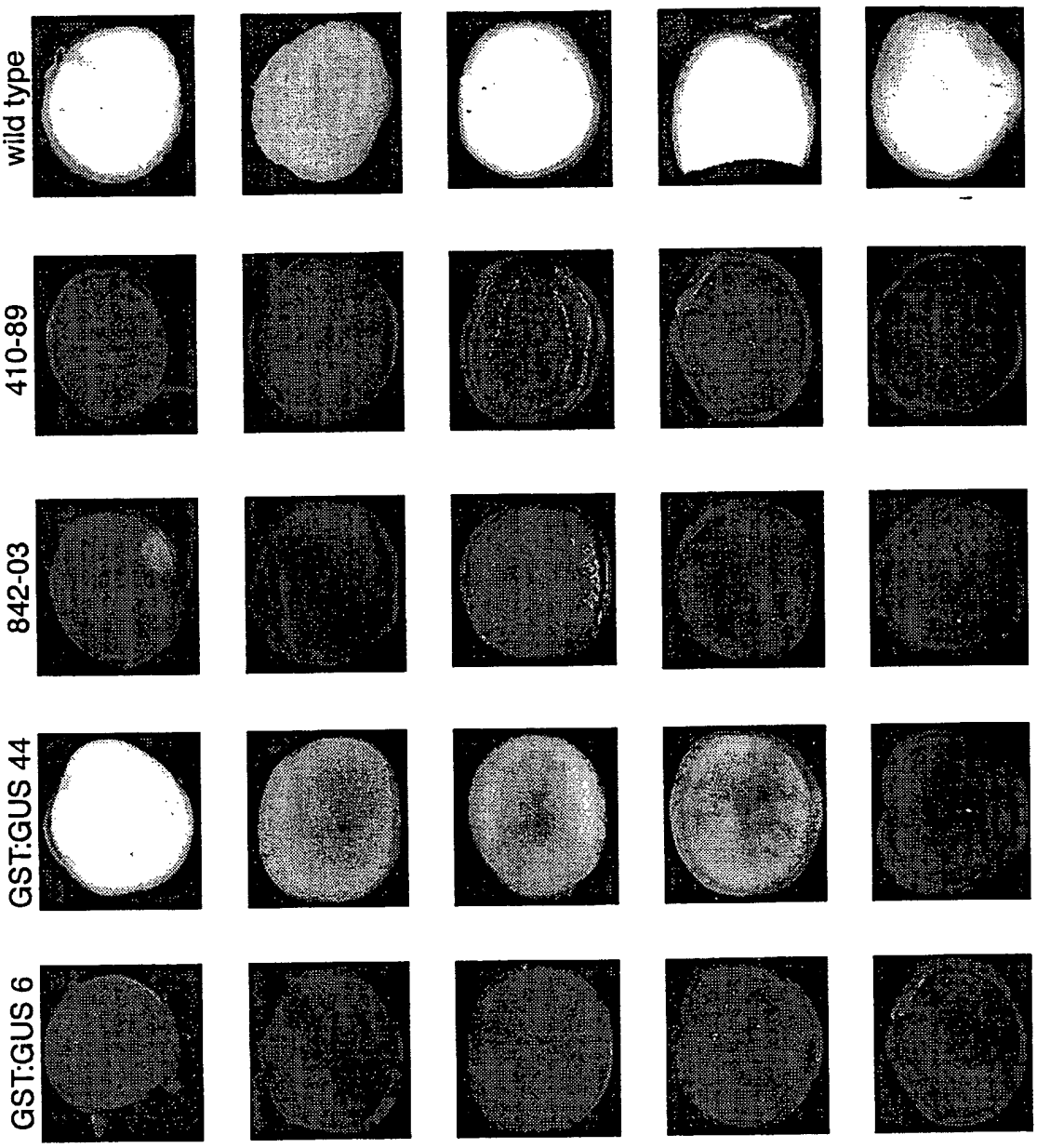

wild type
410-89
842-03
GST:GUS 44
GST:GUS 6
Week 8
Growing Plants
Week 18
Harvest
Week 23
Storage
Week 35
Eyes Formed
Week 47
Sprouting

Fig.17.

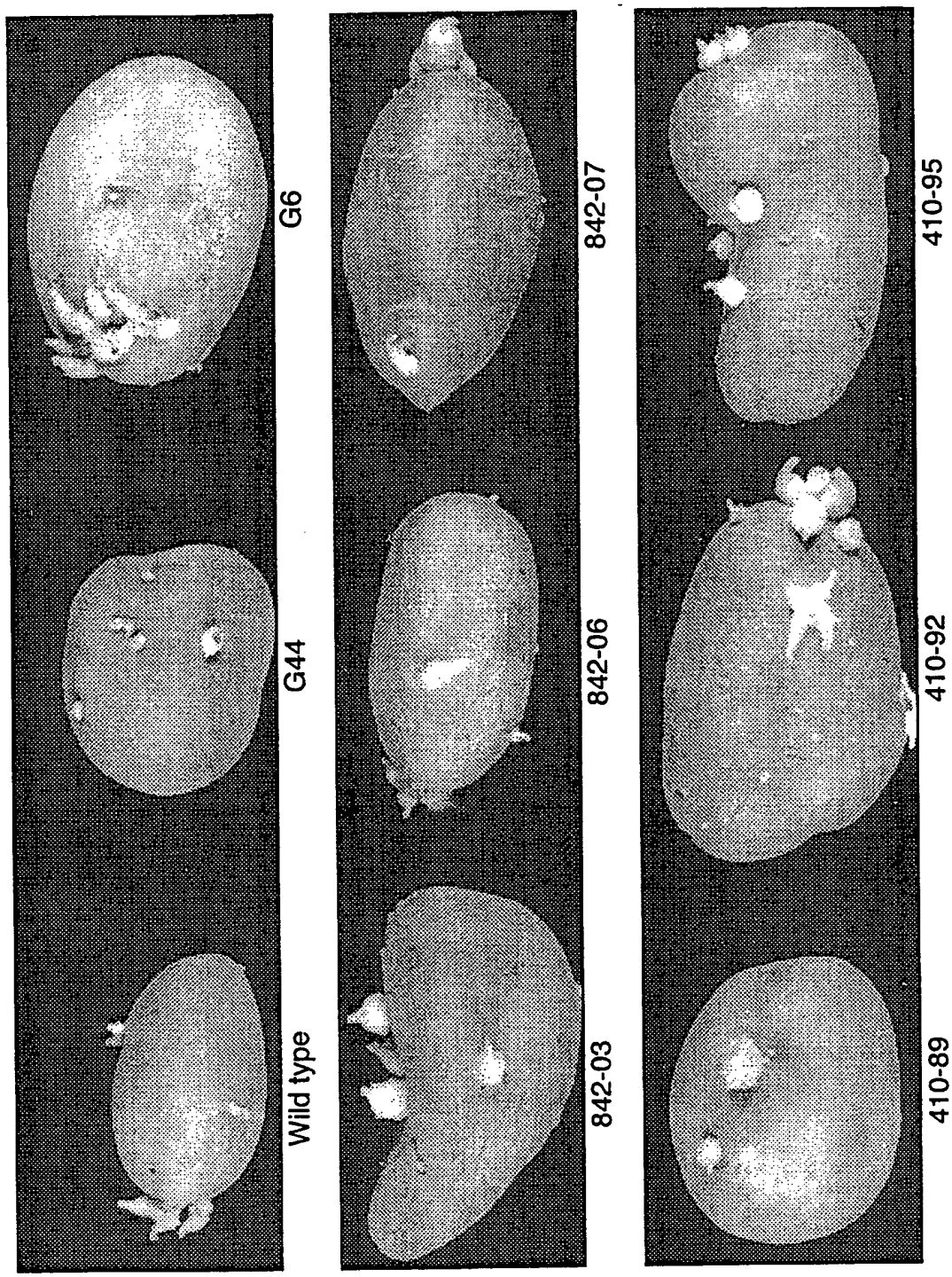
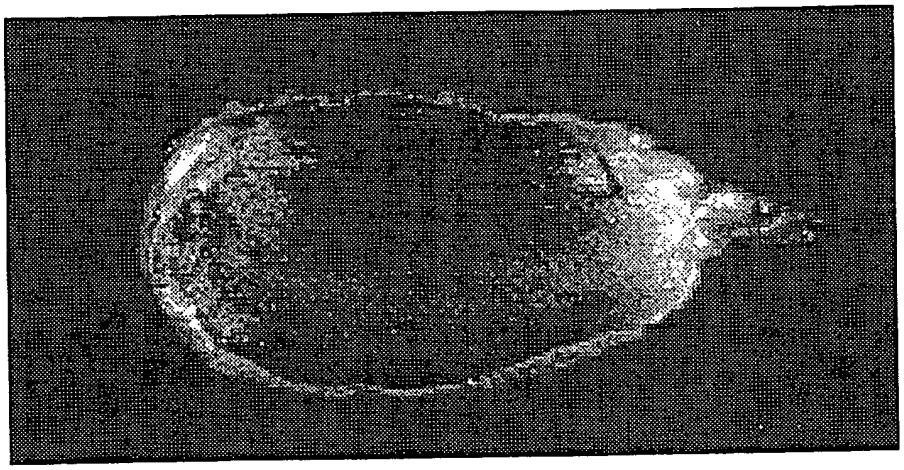


Fig.18.

Fig.19.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03021

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 97 11189 A (BEVAN MICHAEL ;JEPSON IAN (GB); SHEPPARD HILARY (GB); ZENECA LTD () 27 March 1997 (1997-03-27) cited in the application the whole document	1-24
A	WO 93 01294 A (ICI PLC) 21 January 1993 (1993-01-21) cited in the application the whole document	1-24
A	WO 97 44465 A (MONSANTO CO) 27 November 1997 (1997-11-27) the whole document	1-15,24

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 2000	24/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Kania, T

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INTERNATIONAL SEARCH REPORT

Intl ional Application No
PCT/GB 99/03021

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

Inte 'onal Application No
PCT/GB 99/03021

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		EP 0701618 A	20-03-1996
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		JP 8510645 T	12-11-1996
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		DE 4438821 A	25-04-1996
		AU 697450 B	08-10-1998
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